RENEWED PERSPECTIVE ON EFFICACY AND SAFETY OF INHALED MUSCARINIC ANTAGONISTS AS ASTHMA MANAGEMENT THERAPIES

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ABSTRACT

Background: Although muscarinic antagonists are currently used to treat asthma, there remain various characteristics that have yet to be fully elucidated. The other major class of bronchodilators, β_2-agonists, have been extensively studied and through these investigations, it was found that significant tolerance to the bronchoprotective effects of β_2-agonists occurs along with increased allergen responsiveness. These findings have informed clinical use of β_2-agonists to ensure patient safety. For this reason, formulations of long-acting β_2-agonists are combined with inhaled corticosteroids and a recommended upper limit for dosing short-acting β_2-agonists use has been established. By contrast, the regular use effects of muscarinic antagonists on methacholine and allergen responsiveness are not well established. These characteristics can be determined through methacholine challenge testing and allergen inhalation challenges, respectively.

Methods: Two double blind, placebo-controlled, randomized crossover clinical trials were performed. The first trial examined the regular use effect of the inhaled short-acting muscarinic antagonist (SAMA), ipratropium bromide (Atrovent®), on methacholine-responsiveness in twelve well-controlled mild asthmatics. This investigation employed methacholine challenge testing at various timepoints to determine if tolerance developed following 40μg of ipratropium thrice daily for 1-week. The second trial examined the regular use effect of the inhaled long-acting muscarinic antagonist (LAMA), tiotropium bromide (Spiriva® Respimat®), on the allergen-induced early asthmatic response in thirteen well-controlled mild allergic asthmatics. This investigation used an allergen-inhalation challenge and the indirect measures of inflammation: fractional exhaled nitric oxide (FeNO) and sputum differential cell counts (sDCC). Tiotropium was administered 5μg once daily for 1-week.

Results: The first trial found that tolerance to the bronchoprotective effect of ipratropium did not occur after 1-week of regular use. The second trial found that regular tiotropium slightly increased the early asthmatic response to allergen and did not inhibit eosinophil recruitment after allergen.

Conclusion: The absence of tolerance to ipratropium bromide suggest muscarinic antagonists may be safer alternatives for regular use compared to β_2-agonists. This finding is contradicted by the observed increase in allergen responsiveness following regular use of tiotropium. Muscarinic antagonists need to be further evaluated to determine their safety and efficacy as asthma management therapies.

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LIST OF ABBREVIATIONS

Th2	T helper type 2
ILC-2	Type 2 innate lymphoid cells
Th-17	T helper type 17
AHR	Airway hyperresponsiveness
IL	Interleukin
DC	Dendritic cells
MHC	Major histocompatibility complex
TCR	T cell receptor
IgE	Immunoglobulin E
CysLT	Cysteinyl leukotriene
PG	Prostaglandin
EAR	Early asthmatic response
LAR	Late asthmatic response
TSLP	Thymic stromal lymphopoietin
TARC	Thymus and activating regulated chemokine
MDC	Macrophage-derived chemokine
CTS	Canadian Thoracic Society
SABA	Short-acting β_2-agonist
ICS	Inhaled corticosteroids
LTRA	Leukotriene receptor antagonist
LABA	Long-acting β_2-agonist
OCS	Oral corticosteroid
ACh	Acetylcholine
mAChR	Muscarinic acetylcholine receptors

M_1	Muscarinic receptor subtype 1
M_2	Muscarinic receptor subtype 2
M_3	Muscarinic receptor subtype 3
M_5	Muscarinic receptor subtype 5
AChE	Acetylcholine esterase
ChAT	Choline acetyltransferase
nAChR	Nicotinic acetylcholine receptors
COPD	Chronic obstructive pulmonary disease
MBP	Major basic protein
SAMA	Short-acting muscarinic antagonist
pMDI	Pressurized metered dose inhaler
FEV_1	Forced expiratory volume in 1-second
MCh	Methacholine
MCT	Methacholine challenge testing
MCh PD_{20}	Provocative dose of methacholine required to elicit a 20% fall in FEV_1
IV	Intravascular
LAMA	Long-acting muscarinic antagonist
OVA	Ovalbumin
TLC	Total lung capacity
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
NHLBI	National Heart, Lung and Blood Institute
MCh PC_{20}	Provocative concentration of methacholine causing a 20% fall in FEV_1
EAR PD_{20}	Early asthmatic response provocative dose of allergen causing a 20% fall in FEV_1

EAR PC_{20}	Early asthmatic response provocative concentration of allergen causing a 20% fall in FEV_1
SPT	Skin prick testing
STE	Skin titration endpoint
AIC	Allergen inhalation challenge
NO	Nitric oxide
FeNO	Fractional exhaled nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
iNOS	Inducible nitric oxide synthase
eNOS	Epithelial nitric oxide synthase
Ca^{2+}	Calcium
ATS	American Thoracic Society
DTT	Dithiothreitol
PBS	Phosphate buffered saline
sDCC	Sputum differential cell counts
CJRCCSM	Canadian Journal of Respiratory, Critical Care and Sleep Medicine
COVID-19	2019 Novel coronavirus
IB	Ipratropium bromide
PPB	Parts per billion
Tio	Tiotropium bromide

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Asthma

1.1.1 Overview

Asthma is a respiratory disease characterized by airway hyperresponsiveness (AHR) to various stimuli, reversible airflow obstruction, chronic airway inflammation, and tissue remodelling. Asthma affects 2.5 million Canadians and up to 18% of populations worldwide (*Global Initiative for Asthma*, 2021; Statistics Canada, 2019). Common asthma triggers include allergen, exercise, smoke, and cold air which act in the airway to induce narrowing, causing airflow obstruction (O' Byrne et al., 2009). Diagnosis commonly occurs following a clinical history of wheezing, cough, chest tightness and/or dyspnoea accompanied by a favourable response to asthma management therapies (Coates et al., 2017; O' Byrne et al., 2009) and may be confirmed by demonstration of variable airflow obstruction or AHR to inhaled stimuli that act directly on airway smooth muscle (e.g. methacholine). However, the heterogenous nature of asthma complicates treatment, management, and diagnosis. Attempts have been made to categorize clinically similar asthmatic patient groups into asthma phenotypes (Miles, 2012). These phenotypes include allergic, non-allergic, adult-onset, persistent airflow limitation and asthma with obesity (*Global Initiative for Asthma*, 2021). However, these clinical patterns do not always appear to be directly linked to pathologic features and specific treatment response. Instead, focus should be on the underlying disease mechanisms contributing to an individual's asthma in order to provide targeted therapy. Progress has been made in endotyping asthma into subgroups based on pathophysiological disease mechanisms to allow for specific targeted therapies (Côté et al., 2020). This thesis will focus on the general population of mild asthmatics and the sub-population of allergic asthmatics.

1.1.2 General Pathophysiology

Airway inflammation was identified to be central to asthma pathogenesis, which shifted asthma therapies to an anti-inflammatory focus (O' Byrne et al., 2009). However, the type of inflammation present can vary (Hargreave & Nair, 2009). The sputum and bronchial wall of asthmatics contains inflammatory granulocytes involved in the underlying airway inflammation, such as eosinophils and neutrophils. Sputum cell samples may be used to identify inflammatory subtypes in asthmatics and are most often comprised of macrophages, lymphocytes, and

contaminating squamous epithelial cells in addition to the inflammatory granulocytes (Figure 1.1). Eosinophilic inflammation (Figure 1.1 Panel A) tends to occur with an overexpression of T helper type 2 (Th2) lymphocytes and type 2 innate lymphoid cells (ILC-2) (Khalaf et al., 2019). This inflammatory subtype is associated with allergic asthma, although many non-allergic asthmatics also have eosinophilic inflammation. Non-eosinophilic or neutrophilic (Figure 1.1 Panel B) asthma may be driven by T helper type 17 (Th-17) lymphocytes. Less common are mixed neutrophilic/eosinophilic inflammation and paucigranulocytic asthma where few inflammatory cells are present (Papi et al., 2018). Different chemical mediators are released by inflammatory cells and can cause tissue damage leading to airway remodelling, heightened airway responsiveness and chronic airflow limitation (Hargreave & Nair, 2009).

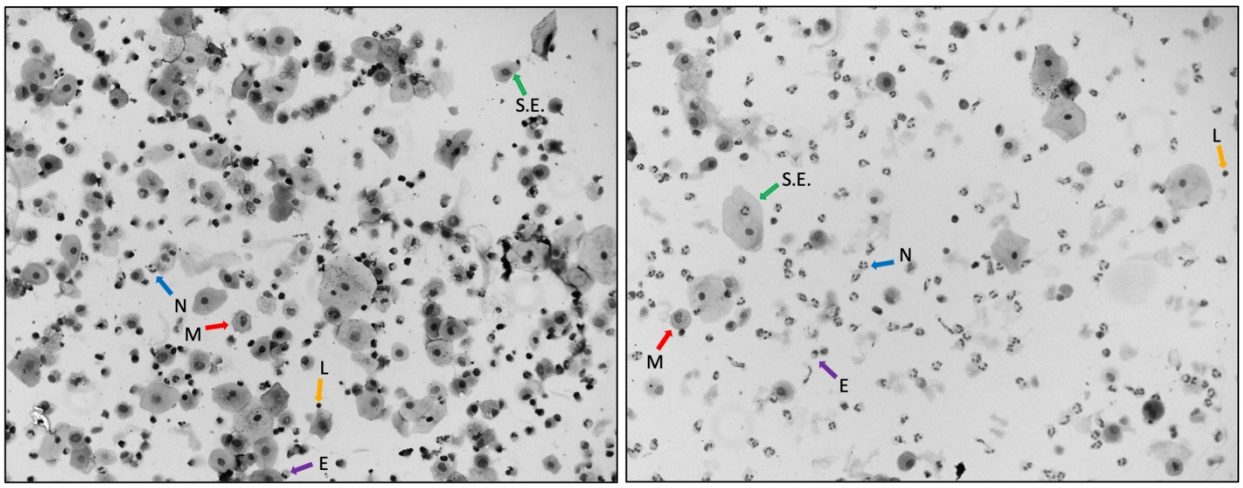

Figure 1.1 *Examples of sputum cell samples for eosinophilic inflammation (Panel A) and neutrophilic inflammation (Panel B). Both samples contain macrophages (red), lymphocytes (orange), eosinophils (purple), neutrophils (blue) and contaminating squamous epithelial cells (green). Although both samples contain all cells, the proportion of eosinophils is much greater in A and that of neutrophils is much greater in B.*

AHR can occur from hypercontractile airway smooth muscle in response to various stimuli (Papi et al., 2018). Certain triggers may initiate worsening of airway inflammation and asthma exacerbations, including allergens and viruses (Mims, 2015). When an asthma exacerbation occurs, airway swelling, secretions and smooth muscle constriction contribute to airway obstruction. Variability in obstruction is defined by both improvement and/or deterioration in symptoms and lung function over time (*Global Initiative for Asthma*, 2021). Progression to

incompletely reversible airflow obstruction signals that structural changes may have occurred, collectively referred to as remodelling (Hargreave & Nair, 2009).

Airway remodelling includes epithelial damage, cilial dysfunction, goblet cell hyperplasia, mucous gland metaplasia and increased airway smooth muscle mass (Papi et al., 2018). Pathological changes to the airway mucosa may lead to increased mucous production and increased mucous in the airway lumen (Mims, 2015).

No single cause of asthma has been identified. However, it has been long understood that there is a genetic component (Miles, 2012). Several polymorphisms exist in genes for airway epithelial barrier function and immune response which may be contributing factors to asthma pathogenesis (Papi et al., 2018). Environmental factors including pollution, allergen, infection and social stress are also considered as potential causes (Apter, 2010). It is likely that an array of genetic and environmental factors influence asthma development.

1.1.3 Allergic Asthma

Pollens, animal antigens and house dust mite were linked to airway disease in the late 19th century and allergens are now recognized as the most important cause of asthma (Cockcroft, 2014). The majority of asthmatics are allergic and atopic children are at an increased risk of developing asthma (Gauvreau et al., 2015). Clinically, allergic asthma includes sensitization to environmental allergens and a correlation between allergen exposure and symptom development (Schatz & Rosenwasser, 2014). While allergic asthma can arise at any age, onset in childhood is more likely to occur. The spectrum of allergic asthma can range from mild to severe. Seasonal sensitivities may lead to individual variations in allergic asthma severity based on the presence of environmental allergens. Hence the term, hay fever, which describes allergic symptoms coinciding with haying season (Gauvreau et al., 2015).

Atopic (or allergic) asthma is considered a type 2 inflammatory disease mediated by T-helper CD4+ lymphocytes and type 2 inflammatory cytokines: interleukin-4 (IL-4), IL-5, IL-9 and IL-13 (Burks et al., 2020). In allergic asthmatics, inhaled allergen induces airway responses via cellular inflammatory cascades that lead to bronchoconstriction. At mucosal barriers including the airway epithelium, an array of immune cells survey the environment in order to provide innate and adaptive immunity as needed (Bosmans et al., 2017). Dendritic cells (DC) are

antigen-presenting cells responsible for processing and presenting antigen to prime naïve T lymphocytes. DC take up antigen via receptor-mediated endocytosis in the case of the major cat antigen Fel d 1, constitutive micropinocytosis for some pollens or phagocytosis for particulate allergens. Any antigens taken up by DC accumulate in the endocytic compartment where they are cleaved into immunogenic peptides and loaded on major histocompatibility complex (MHC) class II molecules. The T cell receptor (TCR) on CD4+ T lymphocytes recognizes antigens in the context of the MHC class II molecules. Through this interaction (Fig. 1.2), DC stimulate naïve T lymphocytes to mature into Th2 cells that produce a variety of type 2 inflammatory cytokines leading to immunoglobulin E (IgE) production, eosinophil recruitment, mast cell development, goblet cell hyperplasia and AHR (Gauvreau et al., 2015). The binding of allergen-specific IgE to FcεRI receptors on the surface of mast cells and basophils initiates allergen-induced airway responses (Fig. 1.3). Cross-linkage of IgE by allergen causes mast cell degranulation of preformed mediators including histamine and the activation of eicosanoid pathways to produce prostaglandins (PG) like PGD_2 and cysteinyl leukotrienes (CysLTs) including LTC_4, LTD_4 and LTE_4.

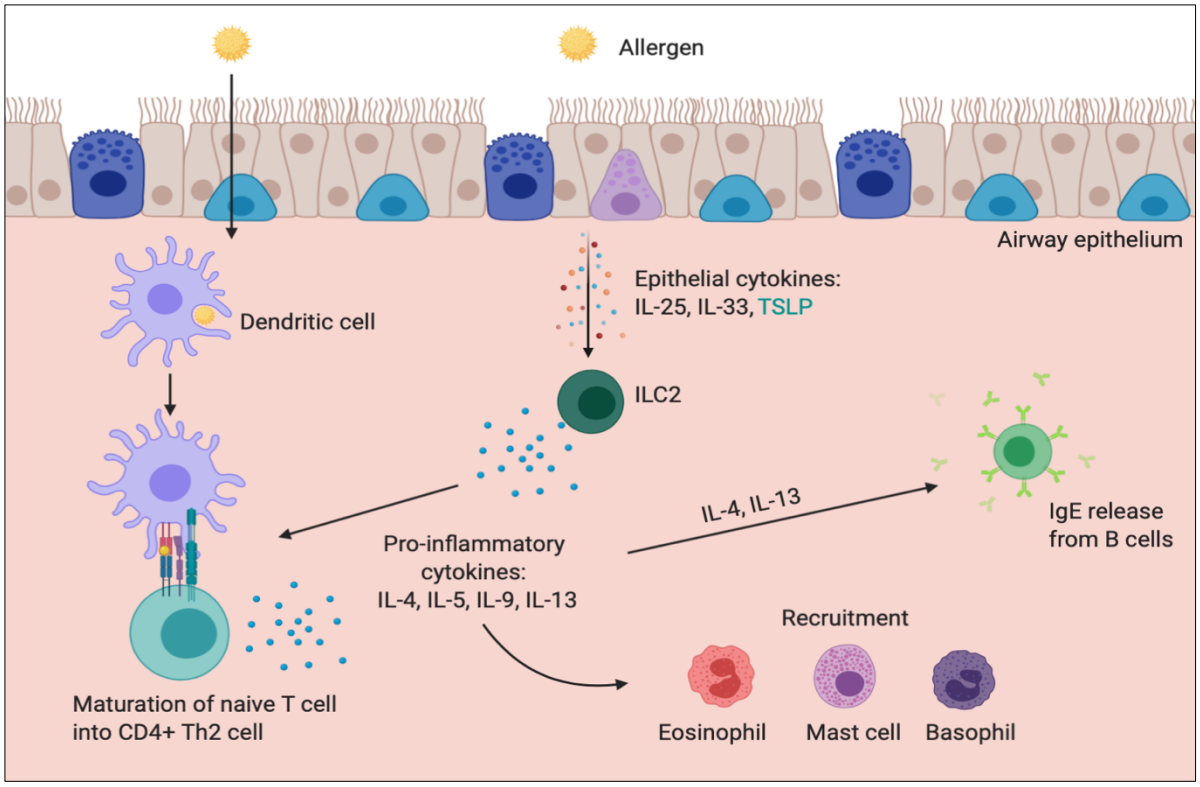

Figure 1.2 *Th2 cellular responses to allergen leading to recruitment of inflammatory cells. Adapted from descriptions by Bosmans et al.*

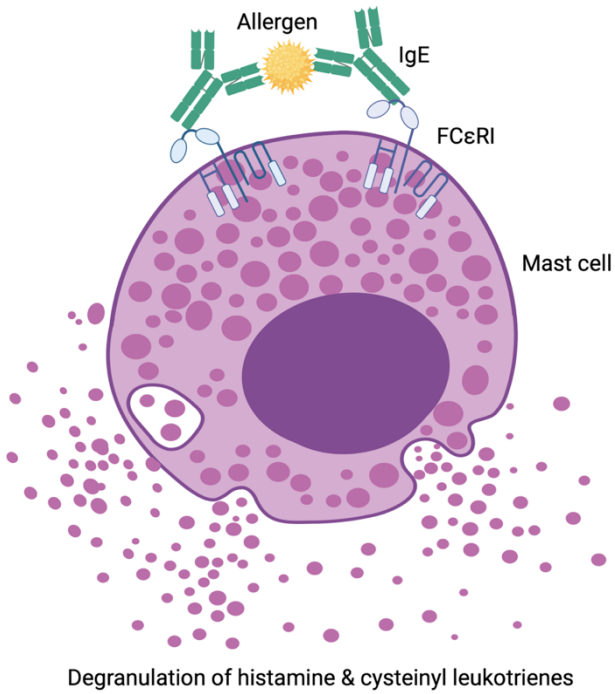

Figure 1.3 *IgE-mediated release of bronchoconstricting mediators during early asthmatic responses to allergen.*

Histamine and CysLTs are responsible for allergen-induced bronchoconstriction, which can occur as an early asthmatic response (EAR), late asthmatic response (LAR) or both, known as a dual response. The EAR develops shortly after allergen exposure and is maximal after 10- to 20-minutes (Boulet et al., 2007; Cockcroft, 2014; Marciniuk et al., 2021). However, the LAR is a recurrence of bronchoconstriction that can manifest between 3 to 8-hours after allergen inhalation. Approximately 60% of adults with allergic asthma are dual responders who experience both the EAR and LAR (Gauvreau et al., 2015). However, this may be an overestimate as only 35% of historical Asthma Research Lab allergic asthmatic participants were dual responders in Saskatchewan (Marciniuk et al., 2021). While cellular mechanisms behind the late response are not fully established, histamine and CysLTs appear to be involved (Davis et al., 2009). Late sequelae including increased AHR to direct acting stimuli and inflammation may occur in addition to the LAR (Cockcroft, 2014). AHR is a defining feature of asthma and increased AHR to histamine and methacholine up to 10-fold and lasting 7-10 days has been documented following allergen inhalation in the laboratory (Boulet et al., 2007; Cockcroft, 2014). This finding is correlated to real world allergen exposure as individuals developing a LAR and AHR to ragweed pollen in the lab are more likely to demonstrate seasonal AHR during ragweed pollen season (Boulet et al., 1983; Cockcroft, 2014). Allergen-induced airway inflammation associated with the LAR is defined by an increase in airway inflammatory cells,

demonstrated by bronchoscopy, bronchoalveolar lavage and induced sputum differential cell counts (Gauvreau et al., 2015). Marked airway eosinophilia occurs in dual and isolated late responders (Cockcroft, 2014). Airway basophils and less consistently, airway neutrophils can also increase following allergen but to a lesser extent than eosinophils (Gauvreau et al., 2015).

Initiation of allergen-induced airway inflammation occurs when crosslinking of allergen-specific IgE on mast cells (Fig. 1.3) induces the release of inflammatory cytokines, IL-3, IL-4, IL-5, and IL-6 (Gauvreau et al., 2015). Proinflammatory cytokines induce inflammation through various mechanisms summarized in Table 1.1. In addition, release of CysLTs from mast cells contributes to inflammation by promoting eosinophil survival (Lee et al., 2000), indirect chemotaxis by stimulating the production of chemoattractants (Peters-Golden et al., 2006) and direct chemotaxis by LTE_4 (Gauvreau et al., 2001). Isolated early responders, compared to dual responders, show smaller increases in airway inflammatory cells despite a similar magnitude of acute bronchoconstriction following allergen inhalation. Therefore, mast cell degranulation cannot be the only mechanism responsible for airway inflammation. The allergen-induced inflammatory response could also occur with the activation and maturation of DC in response to the epithelial cytokine, thymic stromal lymphopoietin (TSLP) (Levine & Wenzel, 2010). DC are the most potent antigen-presenting cell in the lung, playing a major role in immune responses (Gauvreau et al., 2015). This is confirmed in mouse models of allergic asthma that demonstrate allergen induced Th2 sensitization of DC and subsequent eosinophilic airway inflammation.

Regardless of phenotype, most asthmatics are predisposed towards type 2 airway inflammation based on the presence of airway alarmins including TSLP, IL-25 and IL-33 (Gauvreau, Sehmi, et al., 2020; Schatz & Rosenwasser, 2014). TSLP is released by airway epithelium in response to aggravating environmental factors, including allergen (Gauvreau, Sehmi, et al., 2020). Certain inflammatory disorders including asthma and atopic dermatitis show dysregulated production of TSLP and elevated TSLP expression in asthma is correlated with the degree of airway obstruction and disease severity. ILC2 are responsive to these alarmins and secrete high amounts of type 2 cytokines (Gauvreau, Sehmi, et al., 2020; Scanlon & Mckenzie, 2012). The role of ILC2 in the lung has not been fully elucidated; however, their potential to drive type 2 responses suggests ILC2 are involved in driving type 2 inflammation. This is

illustrated by elevated ILC2 in severe asthma and persistent eosinophilia when compared to mild asthma (Gauvreau, Sehmi, et al., 2020).

Table 1.1 *Cytokines involved in allergic airway responses.*

Cytokine	Function
IL-3	Mast cell growth factor; prolongs eosinophil survival (Burks et al., 2020; Marshall et al., 1989).
IL-4	Increases IgE production due to B cell class switching; causes differentiation and proliferation of T lymphocytes; indirectly increases iNOS expression; increases epithelial permeability (Gauvreau et al., 2015; Levine & Wenzel, 2010; Wenzel et al., 2007).
IL-5	Eosinophilopoietin: differentiation, maturation, and survival of eosinophils (Burks et al., 2020).
IL-6	Immunoglobulin secretion factor (Marshall et al., 1989).
IL-9	Mast cell development and stimulates production of mast cell cytokines and chemokines (Burks et al., 2020).
IL-13	Induces goblet cell hyperplasia leading to mucus overproduction; enhances non-specific AHR; indirectly increases iNOS expression; increases epithelial permeability (Gauvreau et al., 2015; Levine & Wenzel, 2010; Wenzel et al., 2007).
TSLP	Alarmin: Central regulator of Th2 immune responses (Gauvreau, Sehmi, et al., 2020).
IL-25	Alarmin: Drives Th2 immune deviation through action on lymphocytes and ILC2 (Burks et al., 2020).
IL-33	Alarmin: Acts as a transcription factor to enhance the Th2 inflammatory response and a chemoattractant for Th2 cells (Burks et al., 2020).

Abbreviations: IgE: immunoglobulin E; iNOS: inducible nitric oxide synthase; AHR: airway hyperresponsiveness; Th2: T-helper type2.

Of particular interest is the role of TSLP in driving dendritic cell-mediated T lymphocyte differentiation. TSLP can induce the production of Th2-attracting chemokines including thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) (Ito et al., 2005). In addition, TSLP increases dendritic cell expression of costimulatory molecules: CD40, CD80, CD86, CD83, HLA-DR and OX40L (Guo et al., 2010; Ito et al., 2005). The OX40-OX40L interaction between DC (OX40L) and T lymphocytes (OX40) plays an important role in triggering Th2 cell immune responses in both human and murine models. Furthermore, when CD4+ T lymphocytes are activated by TSLP-primed DC, the

T lymphocytes are stimulated to produce large amounts of type 2 cytokines. Therefore, TSLP polarizes DC towards a Th2-promoting profile with major implications in allergic asthma (Gori et al., 2017).

1.1.4 Asthma Management

A range of pharmacological treatments targeting various aspects of asthma pathophysiology are available and generally fall into one of two categories: rescue or controller. Rescue treatments relieve acute bronchoconstriction and include bronchodilators such as β_2-agonists (e.g. salbutamol). Controller treatments tend to target airway inflammation and include inhaled corticosteroids (e.g. budesonide). Treatment regimens are divided into tracks or steps based on asthma severity (*Global Initiative for Asthma*, 2021). However, asthma severity is classified by the intensity of treatment required to maintain disease control (Yang et al., 2021). This complicates initial treatment as severity can only be determined retrospectively after treatment has been initiated. While very mild/mild asthma severity implies low risk of morbidity and mortality, asthma exacerbations can cause death in mild asthmatics. For this reason, it is extremely important that disease control is maintained with asthma treatments. The 2021 Canadian Thoracic Society (CTS) guidelines suggest very mild asthma is treated with short-acting β_2-agonist (SABA) as needed no more than twice a week. Previous recommendations suggested mild asthma could also be controlled with SABA as needed and occasional low dose inhaled corticosteroids (ICS) during mild episodes of poor control. However, given the risk of severe exacerbations, it is now suggested that mild asthmatics receive daily low dose ICS to maintain control over inflammation and SABA as needed to relieve episodes of acute bronchoconstriction. Alternatively, mild allergic asthmatics may be treated with daily leukotriene receptor antagonist (LTRA) instead of ICS and mild asthmatics may use combination therapy of ICS/long acting-β_2-agonist (LABA) as needed for symptoms. Asthma is considered well-controlled when daytime symptoms occur $\leq$ 2 days/week, mild nighttime symptoms occur $<$ 1 night/week and SABA reliever is required $\leq$ 2 doses weekly.

Treatment of severe and uncontrolled or difficult-to-control asthma may require different approaches and has been the subject of many clinical investigations (Côté et al., 2020). While the use of oral corticosteroids (OCS) is usually effective, monoclonal antibodies (e.g. anti-

IgE, anti-IL-5) and long-acting muscarinic antagonist add on therapy (e.g. tiotropium bromide) have also proved beneficial in this population and may be preferred to avoid OCS side effects. Given the indication and use of tiotropium bromide in severe asthma, it becomes particularly important to further understand the role of muscarinic signalling and blockade in asthma.

1.2 Airway Muscarinic Signalling

Muscarinic antagonists have been used for respiratory problems dating back centuries to Ayurvedic healers in India (Schlueter, 1986; Williams & Rubin, 2018). In 1802, asthma cigarettes were formulated with muscarinic antagonists and marketed for the treatment of asthma (Herxheimer, 1959). Muscarinic antagonists exert their action by inhibiting muscarinic receptors of the airways to induce bronchodilation. Airway cholinergic signalling may have a role in the pathogenesis of asthma and thus cholinergic modulation may be an effective management mechanism.

1.2.1 Neuronal Cholinergic Signalling

The bronchoconstrictor role of acetylcholine via parasympathetic nervous system signalling has been well established (Kistemaker et al., 2012). Acetylcholine (ACh) is released from the vagal parasympathetic preganglionic fibers onto peri-bronchial ganglia to stimulate subsequent ACh release from the post-ganglionic fibers onto airway smooth muscle and submucosal glands (Novelli et al., 2012). Cholinergic innervation occurs throughout the airways but is greatest in the larger airways (White, 1995). Post-ganglionic neuronal signalling uses muscarinic ACh receptors (mAChR) that are G-protein coupled and exist in 5 subtypes, M_1-M_5 (Nizri & Brenner, 2013). Muscarinic receptors mediate neuronal cholinergic effects on the airways via the M_1, M_2 and M_3 subtypes. The excitatory M_1 and M_3 receptors promote ACh release through G-protein coupling to G_q/G_{11} and subsequent activation of phospholipase C and phosphatidylinositol turnover to increase intracellular calcium (Williams & Rubin, 2018). M_1 receptors facilitate neural transmission on the parasympathetic ganglia, while M_3 receptors mediate cholinergic action on the smooth muscle, vascular endothelium, and submucosal glands of the airways (Novelli et al., 2012). Neuronal cholinergic signalling results in bronchoconstriction of airway smooth muscle and mucus secretion. Modulatory M_2 receptors mediate inhibitory effects on ACh release through G_i/G_0 coupling that inhibits adenylate cyclase and induces hyperpolarization of

the cell membrane (Williams & Rubin, 2018). The presence of M_2 receptors on the post-ganglion endings of vagal cholinergic fibers inhibits presynaptic ACh release in response to increased synaptic concentrations of ACh (Novelli et al., 2012; Williams & Rubin, 2018). Acetylcholine esterase (AChE) is highly effective at degrading neuronal ACh to prevent it from diffusing as a signal molecule (Wessler & Kirkpatrick, 2008).

Pathogenic neuronal cholinergic signalling in asthma has been characterized as AHR to inhaled cholinergic agonists (e.g. methacholine) and in some cases, enhanced cholinergic tone and mucus hypersecretion (Novelli et al., 2012). However, increasing evidence has implicated additional cholinergic signaling from non-neuronal cells in immune system modulation.

1.2.2 Non-Neuronal Cholinergic Signalling

Cholinergic receptors and signal transduction pathways can be used by non-neuronal cells as a mode of communication (Wessler & Kirkpatrick, 2008). The airway non-neuronal cholinergic system consists of mast cells, leukocytes, macrophages, DC, and airway epithelial cells. Much of the research on the non-neuronal cholinergic signalling employed by immune cells stems from *in vitro* murine models and *ex vivo* human tissue data. Given the relevance of these data to human allergic asthma, cholinergic signalling among lymphocytes, DC, mast cells, airway epithelium and eosinophils will be focussed on.

Release of ACh from non-neuronal cells mediates paracrine and autocrine regulatory functions that are not fully elucidated to date (Wessler & Kirkpatrick, 2008). Cells with the ability to produce and release ACh have been identified by immunohistochemistry demonstrating the presence of choline acetyltransferase (ChAT), the main ACh synthesis enzyme (Wessler & Kirkpatrick, 2001). While various immune cells may produce ACh, the ability of T lymphocytes and DC to synthesize and release ACh becomes particularly important in the polarization of DC and T lymphocytes to a Th2 profile. Upon stimulation with antigen, T lymphocytes are triggered to express ChAT and M_5 receptors (Kawashima, 2004). Resting DC also do not express ChAT; however, expression can be induced by endotoxin stimulation (Kawashima et al., 2007). Therefore, it is theorized that cholinergic signalling may be activated during antigen presentation and the ACh released by DC and T lymphocytes acts on their own mAChR and nicotinic ACh receptors (nAChR) to modulate functions.

Expression of mAChRs and nAChRs differs among phenotypic cell functions (Wessler & Kirkpatrick, 2008). The α7 nAChR seems to mediate anti-inflammatory effects while mAChR provide a proinflammatory signal (Gori et al., 2019). DC are key players of innate type 2 immunity that influence subsequent development of adaptive immune responses (Bosmans et al., 2017). DC express cholinergic receptors and evidence suggests that cholinergic signals modulate the activity of these cells. Murine DC can express the α7 nAChR subtype and all five mAChR subtypes. An *ex vivo* human dendritic cell investigation showed stimulation of DC with ACh via mAChR increased the expression of OX40L and increased the production of chemokines: MDC/CCL2 and TARC/CCL17 (Gori et al., 2017). Lymphocyte proliferation and production of IL-4, IL-5 and IL-13 also increases with ACh-primed DC, suggesting that ACh polarizes DC towards a Th2 promoting profile in a similar mechanism to TSLP. In fact, ACh and TSLP synergistically increase OX40L, CD83 and HLA-DR when incubated together. This enhancing effect could be due to upregulated expression of the M_3 receptor by TSLP. These cholinergic effects are mediated by the M_3 receptor on murine DC (Gori et al., 2019). Therefore, M_3 is considered the main receptor involved in ACh-activation of DC.

Given the role of mast cells in airway responses to allergen, cholinergic signalling mechanisms influencing mast cell function are of potential importance in allergic asthma. Both mAChR and nAChR have been identified on mast cells (Bosmans et al., 2017). Mast cells reside in the airway mucosa and can become activated by inhaled allergens causing degranulation of histamine and CysLTs. However, M_1 stimulation by ACh inhibits the release of histamine from mast cells in *ex vivo* human bronchi (Reinheimer et al., 2000). In addition, the physiological pathway for mast cell activation via IgE crosslinking at the FcεR is effectively inhibited by ACh (Reinheimer et al., 1997). M_1 receptor blockade on mast cells would remove this cholinergic inhibition and therefore, may enhance histamine release in response to allergen. This is not conserved in murine models and thus murine data on non-neuronal cholinergic signalling should be interpreted with caution (Reinheimer et al., 2000). In fact, murine models demonstrate the opposite effect with increased mast cell sensitivity to ACh in the presence of IgE, causing cholinergic histamine release (Masini et al., 1985). Since histamine is partially responsible for allergen-induced bronchoconstriction, therapies should aim to reduce histamine release. For

example, antihistamines are effective at mast cell stabilization and protection against allergen-induced asthmatic responses (Davis et al., 2009).

Eosinophils are considered central effector cells in allergic inflammation (Bosmans et al., 2017). Accumulation of eosinophils in the bronchial wall is regulated by several mechanisms including the release of chemoattractant factors that signal egression from the bloodstream to this secondary site (Håkansson et al., 1990; Koyama et al., 1998). In bovine bronchial epithelial cell models, ACh stimulates the release of eosinophil chemotactic factors (Koyama et al., 1998) and therefore, cholinergic signalling at structural airway epithelium may enhance eosinophil infiltration. It is suggested that bronchial epithelium is involved in initiating inflammatory responses, perhaps doing so with an efficient autocrine cholinergic signalling pathway (Gosens et al., 2006; Proskocil et al., 2004). In addition to mediating chemotaxis, cholinergic signalling may alter eosinophil function as eosinophils express M_1 receptors under certain conditions, such as chronic obstructive pulmonary disease (COPD). However, the effect of ACh on eosinophils is unknown (Profita et al., 2005).

Finally, the role of immune cell crosstalk with nerves is an important area of interest. A bidirectional relationship exists between the nervous system and immune system and likely involves cholinergic signalling (Bosmans et al., 2017). Neurons express cytokine receptors and cytokines may provide simultaneous modulation of immune and neuronal function (Chavan et al., 2017). Both allergen inhalation and airway inflammation can phenotypically change vagal afferent nerves leading to bronchospasm by previously inert stimuli (Mazzone & Undem, 2016). While these neuro-immune interactions are highly complex, they likely contribute to asthma pathophysiology. For example, eosinophil-nerve interactions are demonstrated to occur under inflammatory conditions in the lung (Bosmans et al., 2017). During inflammatory states, eosinophils may localize to airway nerves by recognizing neuronal adhesion molecules and undergo subsequent activation (Kingham et al., 2003). Activated eosinophils release major basic protein (MBP), an antagonist at M_2 receptors that can cause a loss of M_2 function. An impaired autoregulatory role of M_2 in decreasing ACh release can cause increases in vagally induced bronchoconstriction and mucous production. Thus, eosinophils may be recruited by non-neuronal cholinergic signalling and subsequently impair neuronal cholinergic signalling mechanisms.

1.2.3 Short-Acting Muscarinic Antagonist: Ipratropium Bromide

Ipratropium bromide (Atrovent®), previously called SCH 1000, was the subject of over 1,000 clinical articles in the first 15-years of its release (Schlueter, 1986). It is a short-acting muscarinic antagonist (SAMA) administered therapeutically via the pressurized metered-dose inhaler (pMDI) and the first modern inhaled muscarinic antagonist for relieving bronchoconstriction (Davis et al., 2018; Williams & Rubin, 2018). Modern muscarinic antagonists are synthetic derivatives of alkaloid compounds that are modified to have little systemic absorption and blood-brain barrier translocation (Williams & Rubin, 2018). For this reason, inhaled muscarinic antagonists exhibit very few systemic side effects at therapeutic doses and have negligible effects on the heart (Schlueter, 1986). Inhibition of salivary secretions and dry mouth may occur at doses higher than clinical recommendations (Cugell, 1986). Inhaled muscarinic antagonists bind both to the active site and to an allosteric site, changing the active site conformation (Williams & Rubin, 2018). Ipratropium has non-selective affinity for muscarinic receptors of the airways including M_1, M_2 and M_3 subtypes. While M_1 and M_3 receptor blockade induces bronchodilation, M_2 blockade increases release of ACh from vagal endings, counteracting the bronchodilator effect to some extent (Sposato et al., 2005). Therefore, the non-selective nature of ipratropium bromide may limit its use as a bronchodilator.

Bronchodilation mediated by muscarinic antagonists occurs primarily in larger airways, compared to β_2-agonist bronchodilators which act on both small and large airways (Ingram et al., 1977). A standard dose of ipratropium is 40μg as needed and each actuation of the pMDI delivers 20μg (Davis et al., 2018). The criteria for a significant post-bronchodilator response are generally accepted to be an increase in the forced expiratory volume in 1-second (FEV_1) by 12-15% and 200mL (Pellegrino et al., 2005). Changes in $FEV_1 < 8\%$ or < 150mL are within measurement variability. The bronchodilation response to ipratropium has been well documented (Cockcroft et al., 1982; Petanjek et al., 2007; Ruffin et al., 1977). Ipratropium demonstrates a mean increase in FEV_1 by 19.14% in mild to moderate allergic asthmatics (Petanjek et al., 2007). This was replicated in the general asthmatic population with a mean increase in $FEV_1 > 20\%$ (Cockcroft et al., 1982). This relationship is reported to be dose-dependent with 80μg of ipratropium inducing double the bronchodilation (36.3% versus 17.0% mean increase in FEV_1) of a 40μg dose (Lulling et al., 1981). Bronchodilation onsets within 15-minutes but has been

reported to begin as quickly as 30-seconds and lasts 4 to 6-hours (Pakes et al., 1980). The maximal effect is reached after 1.5-hours. Fast onset of bronchodilation is an important property of SABA and SAMA required to treat acute bronchoconstriction (Papiris et al., 2009). Therefore, the bronchodilator action of ipratropium has been frequently compared to the SABA, salbutamol (Ventolin®). Salbutamol induces bronchodilation rapidly and is the first-line rescue treatment for acute symptomatic bronchospasm (Williams & Rubin, 2018). Salbutamol tends to exhibit greater bronchodilator efficacy compared to that of ipratropium in asthmatics (Novelli et al., 2012). However, β_2-agonists are known to produce cardiovascular side effects and tremor that some patients may not tolerate (Ruffin et al., 1978; Schlueter, 1986). In that case, ipratropium may be used instead given its low side effect profile. While regular use of ipratropium is expected to elicit tolerance to the bronchodilator effect (Friedman et al., 1983; Emmelin, 1961), clinical investigations have failed to demonstrate this (Cockcroft et al., 1982; Newcomb et al., 1985). Tolerance to bronchodilation is difficult to demonstrate as there is an upper limit to maximal airway dilation and with current asthma control guidelines, it is difficult to find asthmatic participants with significant resting bronchoconstriction. Instead, bronchoprotection can serve as a method of measuring tolerance development as it is not limited by a maximal response or by the absence of resting bronchoconstriction.

Bronchoprotection occurs when a drug antagonizes, either by specific antagonism or functional antagonism, stimulus-induced bronchoconstriction. Methacholine is a cholinergic agonist that induces bronchoconstriction by direct stimulation of M_3 receptors on airway smooth muscle (Sposato et al., 2005). Muscarinic antagonists elicit bronchoprotection against methacholine by competitive antagonism. β_2-agonists exhibit bronchoprotection by functional antagonism, protecting against constriction by inducing bronchodilation. Methacholine challenge testing is used to quantitate pharmacological bronchoprotection by calculating the shift in the dose of methacholine required to elicit a 20% fall in FEV_1 (methacholine PD_{20}). Comparing methacholine PD_{20} when untreated to the PD_{20} following drug administration quantifies bronchoprotection (Westbury et al., 2018). The shift in methacholine PD_{20} required to overcome the bronchoprotection by a drug is expressed as a doubling dose shift. A 40μg dose of ipratropium produces bronchoprotection in a 2.8 doubling dose shift (Sposato et al., 2005). This is a dose-response effect as 80μg of ipratropium provides a 5.8 doubling dose shift in

methacholine PD_{20} (Bandouvakis et al., 1981). Bronchoprotection lasts at least 6-hours and is absent by 12-hours following administration of 40μg of ipratropium (Illamperuma et al., 2009). In comparison, single dose (200μg) salbutamol induces, on average, bronchoprotection by about 2.7 doubling doses (Cockcroft et al., 2020).

Regular use of β_2-agonists quickly leads to the development of tolerance to the bronchoprotective effect against methacholine-induced constriction (Bhagat et al., 1995; Kalra et al., 1996; Stewart et al., 2012). Administering 200μg of salbutamol twice daily produces a loss of bronchoprotection after only five doses and is significant following the seventh dose (Stewart et al., 2012). ICS do not prevent tolerance development, suggesting airway inflammation does not play a role (Kalra et al., 1996). In addition, regular use of salbutamol has also been shown to increase airway responses (acute bronchoconstriction at lower doses and increased airway inflammation) to allergen (Cockcroft et al., 1993). While the loss of bronchoprotection against methacholine occurs at low doses (200μg salbutamol daily for 1-week), loss of bronchoprotection against allergen (i.e. increased allergen responsiveness) was only documented to occur after 800μg daily for 1-week, suggesting different mechanisms are responsible (Bhagat et al., 1996). Tolerance to bronchoprotection against methacholine may be due to altered β_2-receptor physiology after repeated β_2-agonist exposure (Bhagat et al., 1995) which could include uncoupling of β_2-receptors from the cell membrane, internalization of the receptor (Cooper & Panettieri, 2008) and/or decreased receptor mRNA stability (Hadcock et al., 1989).

Muscarinic receptor upregulation occurs in *ex vivo* human tissues from the brain and gut following repeated exposure to a muscarinic antagonist (Friedman et al., 1983; Newcomb et al., 1985). Extrapolating this effect to the lung suggests that regular use of muscarinic antagonist bronchodilators may lead to tolerance as a result of M_3 receptor upregulation. One previous study found no change in the bronchoprotective effect of ipratropium against methacholine following 60μg of ipratropium four times daily for 3-weeks (Newcomb et al., 1985). However, rebound hyperresponsiveness to methacholine 24-hours after discontinuation of ipratropium was documented. Various aspects of this study differ from current tolerance investigations and tolerance to the bronchoprotective effect of ipratropium should be re-evaluated.

With emerging evidence on the role of cholinergic signalling in allergic asthma, muscarinic antagonist efficacy in allergic asthma is of great interest. Historically, pre-treatment with muscarinic antagonists including atropine and ipratropium prior to an allergen inhalation challenge produced rather equivocal data. Atropine is a naturally occurring anticholinergic alkaloid and was likely the first pharmacologic agent used for asthma in the western world (Restrepo, 2007). A major finding of these early investigations was that the cholinergic pathway did not appear to be involved to a large extent in the airway obstruction induced by allergen inhalation in allergic asthmatics. Inhalation of 1.2mg/mL of atropine in solution with allergen produced asthmatic responses quantitatively similar to the inhalation of allergen alone (Fish et al., 1977). However, 1.5-2.5 mg of atropine administered intravenously reduced airway resistance following allergen inhalation in five of seven participants (Yu et al., 1972). Various administration routes of atropine have been tested including inhalation, sublingual preparations, and intravascular (IV) injections (Itkin & Anand, 1970). Local inhaled atropine is more effective at blocking cholinergic signalling than systemic IV administration although near toxic doses are required to have anticholinergic effects in the lung when given via the IV route. Systemic atropine use is associated with high rates of classical anti-cholinergic adverse effects and inhaled atropine has poor water solubility when compared to modern synthetic muscarinic antagonists like ipratropium (Novelli et al., 2012). For this reason, atropine is, for the most part, no longer used in the treatment of bronchospasm (Cugell, 1986).

Data on single-dose ipratropium efficacy against allergen-induced responses generally shows protection against the EAR in a portion of study populations, suggesting there may be innate differences in allergic asthmatics that may predispose them to better protection with a muscarinic antagonist. An 80μg dose of ipratropium (via MDI) protected against allergen induced bronchoconstriction, with seven of twelve participants experiencing ≥ 25% reduction in the decrease in FEV_1 following allergen (Cockcroft et al., 1978). The reduction in FEV_1 decrease was significant for the study group. In four participants who developed a LAR, ipratropium produced no inhibition. Perhaps due to insufficient protection by ipratropium during the LAR development given its short-half life (Table 1.2). Ipratropium also significantly reduced the response to histamine inhalation. This finding was confirmed by another investigation where a 40μg dose of ipratropium (via MDI) provided protection against allergen and histamine,

measured by tracking the number of breaths required to produce a fall in FEV_1 of 20% (Clarke et al., 1982). The asthmatic response to grass pollen was fully inhibited in six of ten participants following a single 1mg dose of ipratropium (via MDI) despite increased doses of pollen administered (Orehek et al., 1975). These data are contradicted by the finding that 1mg of nebulized ipratropium had no significant effect on the allergen-induced fall in FEV_1 in a group of six allergic asthmatics (Howarth et al., 1985). However, there was a slightly lower mean maximal fall in FEV_1 following ipratropium versus placebo treatment of 20.6% and 25.5%, respectively. Collectively these results suggest that ipratropium has some protective effect against allergen-induced responses in a portion of allergic asthmatics.

1.2.4 Long-Acting Muscarinic Antagonist: Tiotropium Bromide

Tiotropium bromide monohydrate (Spiriva® Respimat®) is currently the only long-acting muscarinic antagonist (LAMA) approved for use by Health Canada and recommended by the CTS as add-on therapy in asthma (Fitzgerald et al., 2017). The Respimat® inhaler device delivers a mist containing 2.5µg of tiotropium per actuation (*Spiriva® Respimat® Product Monograph*, 2017). Recommended dosing is 2 actuations (5µg) once daily at the same time each day. Tiotropium is kinetically selective for the M_3 receptor but binds to M_1, M_2 and M_3 receptors of the airways (Kistemaker & Gosens, 2015). The dissociation half-life of tiotropium is greatest on the M_3 receptor (Table 1.2), creating a duration of action over 24-hours (Sposato et al., 2008). In asthma, its effective half-life is 34-hours (*Spiriva® Respimat® Product Monograph*, 2017). Given this long duration of action, its use is cautioned in patients with narrow-angle glaucoma and urinary retention.

Table 1.2 Anticholinergic selectivity on muscarinic receptors.

Muscarinic antagonist	**Binding Affinity (-log M)**			**Half-life (hr)**		
	M_1	**M_2**	**M_3**	**M_1**	**M_2**	**M_3**
Ipratropium	9.40	9.53	9.58	0.1	0.03	0.22
Tiotropium	10.80	10.69	11.02	10.5	2.6	27

Adapted from Kistemaker et al [2015].

The indication for tiotropium as an add-on therapy is in severe asthmatics who still experience symptoms while on ICS/LABA combination therapy and have experienced one or more severe asthma exacerbations in the last year (*Spiriva® Respimat® Product Monograph*, 2017). Clinical trials under this indication demonstrated significant lung function improvements in FEV_1 when tiotropium is added-on, compared to ICS/LABA alone. Tiotropium also significantly reduced the risk of exacerbations, including severe exacerbations. The benefits of tiotropium treatment arise after several doses. It is currently not recommended to use tiotropium as a monotherapy in asthma based on a lack of data showing any efficacy. As a monotherapy, a single dose of 10µg of tiotropium provides mild bronchodilation between 5.5-11% from baseline that lasts 24-hours in mild asthmatics (O'Connor et al., 1996). Another investigation found 5µg tiotropium provided no bronchodilation 1-hour after inhalation (Blais et al., 2016). However, a borderline statistically significant increase in FEV_1 by 94mL was present 24-hours after inhalation. These study populations were comprised of mild well-controlled asthmatics who have relatively low airway constriction at rest which may contribute to the low level of bronchodilation achieved.

A single dose (5µg) of tiotropium provides bronchoprotection against methacholine-induced constriction on a magnitude of about 4-doubling doses from baseline which remain statistically, but not clinically significant after 7-days (Blais et al., 2016). Currently there are no data on tiotropium protection against allergen-induced asthmatic responses. However, animal data show promise in tiotropium efficacy against allergen-induced changes. Ovalbumin (OVA)-sensitized and challenged guinea pigs and murine are commonly used for studying inhaled allergen-induced changes in the airways (Smith & Broadley, 2007). In BALB/C mice challenged with OVA, tiotropium prevented the acute inflammatory reaction following allergen challenge and decreased eosinophils in bronchoalveolar lavage fluid (Ohta et al., 2010). Tiotropium also prevented the production of type 2 cytokines including IL-4, IL-5 and IL-13 following allergen exposure. In both rats and mice, tiotropium inhibited the OVA-induced LAR (Raemdonck et al., 2012). This was replicated in OVA-challenged guinea pigs where tiotropium significantly inhibited the LAR, but not inflammatory cell infiltration in bronchoalveolar lavage fluid. However, another study with OVA-challenged guinea pigs found tiotropium partially prevented eosinophilia in the airways and effectively inhibited allergen-induced airway remodelling (Bos et al., 2007).

Collectively, these preclinical data suggest that tiotropium should be investigated clinically in allergic asthma.

The protective mechanism of tiotropium against allergen-induced changes may be blockade of the M_3 receptor that is involved in ACh-polarization of DC to a Th2 profile. An *ex vivo* murine dendritic cell investigation demonstrated that tiotropium is efficacious in preventing ACh-induced increases in inflammatory cytokines (Gori et al., 2019). However, knockout of the M_3 receptor does not change eosinophil or type 2 cytokine levels in OVA-challenged mice (John-Schuster et al., 2017). Inhibiting the polarization of DC towards a Th2 profile in humans by targeting TSLP with therapeutic antibodies has had significant efficacy in protecting against allergen-induced responses (Gauvreau, Hohlfeld, et al., 2020; Gauvreau et al., 2014). An injected anti-TSLP monoclonal antibody partially attenuated both the EAR and LAR, decreased blood and sputum eosinophil counts and decreased fractional exhaled nitric oxide levels (Gauvreau et al., 2014). Recently an anti-TSLP antibody fragment was developed as a dry-powder inhaler and demonstrated similar attenuation of allergen-induced changes (Gauvreau, Hohlfeld, et al., 2020). Perhaps the remaining allergen responsiveness in the TSLP-investigations is due to an intact cholinergic signalling pathway promoting Th2 polarization that could be blocked by muscarinic antagonism. Therefore, the effect of tiotropium on allergic responses should be evaluated, despite conflicting preclinical data. The role of the M_1 receptor in inhibiting histamine release from mast cells could be partially responsible for the mixed effects observed in preclinical investigations as tiotropium binds to, and has a significant half-life on the M_1 receptor (Table 1.2). By contrast, β_2-agonists inhibit mast cell degranulation and show efficacy against inhibiting allergen-induced asthmatic responses in humans (Eiser, 1991; Howarth et al., 1985; Ruffin et al., 1978). However, regular use of β_2-agonists enhances both the EAR and LAR, suggesting airway inflammation may occur with regular β_2-agonist use from mast cell dysfunction (Cockcroft et al., 2007; Cockcroft et al., 1993; Cockcroft et al., 1995). In conclusion, the cholinergic role in asthma is complex and requires further investigation.

1.3 Introduction Summary

Asthma is a heterogenous disorder, complicating treatment, and management. Further understanding of the complex disease mechanisms underlying various asthma endotypes means that therapies can specifically target underlying pathophysiology. The mechanisms behind

allergic asthma have yet to be fully determined, especially the role of cholinergic non-neuronal signalling in human immune responses. Cholinergic signalling mechanisms can be targeted by inhaled muscarinic antagonists, which are available and prescribed for use in asthma based on their bronchodilator effect on airway smooth muscle. However, various properties of muscarinic antagonists have yet to be characterized. For example, little is known on regular use of muscarinic antagonists and whether tolerance develops to their bronchoprotective effect. In addition, the efficacy of LAMA against allergen-induced asthmatic responses has yet to be determined. By contrast, our understanding of the detrimental effects following regular β_2-agonists has improved our knowledge of allergic responses and helped inform clinical use of these therapies.

1.4 Rationale

Bronchodilators including salbutamol and ipratropium protect against inhaled bronchoconstrictor stimuli, such as methacholine. Dose-dependent bronchoprotection against methacholine induced bronchoconstriction has been documented for both ipratropium and salbutamol (Bandouvakis et al., 1981; O'Connor et al., 1992; Sposato et al., 2005). Regular use of β_2-agonists results in tolerance to the bronchoprotective effect (Bhagat et al., 1995; O'Connor et al., 1992). Only one study conducted more than 35 years ago reported the absence of tolerance and the presence of rebound hyperresponsiveness following regular use of ipratropium bromide (Newcomb et al., 1985). This finding should be confirmed or refuted by investigating the effect of daily administration, and subsequent withdrawal, of ipratropium bromide on methacholine responsiveness in asthma.

Recent studies have suggested a non-neuronal cholinergic signalling role in allergic asthma through immune cell modulation. ACh has a modulatory action on muscarinic receptors of DC to promote and induce an allergic or type 2 response (Gori et al., 2019; Gori et al., 2017). It is theorized that muscarinic antagonists could protect the airways by inhibiting the ACh-induced polarization of dendritic cells towards the Th2-profile in addition to inhibiting constriction (Gori et al., 2017). Ovalbumin challenge data from animal models treated with tiotropium show reductions in measures of allergen induced changes including eosinophil recruitment and type 2 cytokine production (Bos et al., 2007; Ohta et al., 2010). There are recent reports of synergism between ACh and TSLP in promoting the Th2 phenotype in *ex vivo* dendritic cell investigations

(Gori et al., 2019; Gori et al., 2017). This synergistic effect can be inhibited by muscarinic receptor antagonism of murine DC. Data documenting anti-TSLP efficacy in the human allergen challenge model shows a significant but not complete reduction in allergen-induced changes (Gauvreau et al., 2014). The residual responses could be due to cholinergic signalling via the muscarinic receptors of immune cells. Collectively, these data suggest that the effects of tiotropium on allergen induced airways responses should be evaluated clinically in humans.

Evaluating the safety of regular use of muscarinic antagonists and their efficacy in allergic asthma could provide valuable insight to inform clinical use of muscarinic antagonists in asthma management.

2.0 METHODOLOGY

This chapter will provide background regarding the purpose and function of each method. The method sub-chapters detail specifics from the clinical studies in Chapters 3 and 4 for performing spirometry, methacholine challenge testing, skin prick testing, allergen inhalation challenges, fractional exhaled nitric oxide measurements, sputum induction and sputum differential cell counts.

2.1 Spirometry

Spirometry measures the maximal amount of air an individual can exhale (and inhale) with maximal effort (Graham et al., 2019) and it is useful for diagnosing and monitoring lung disease. Spirometric maneuvers produce volume and flow data as a function of time. Lung function can be predicted based on age, weight, height, sex, and ethnicity as these factors are often determinants of lung size. A maneuver consists of 4 phases: maximal inspiration to total lung capacity (TLC), a blast of expiration, continued expiration up to 15-seconds, and inspiration back to TLC. Spirometry can generate the FEV_1 and the forced vital capacity (FVC) which are reproducible measures of lung function, provided maximal effort and proper technique are performed.

Variable airway obstruction is a characteristic of asthma that can be demonstrated by spirometry. Obstruction manifests as a reduced ratio in FEV_1 to FVC and/or decreased FEV_1 when compared to predicted values (*Global Initiative for Asthma*, 2021). When performed before and after administration of a rapid-acting bronchodilator, spirometry can confirm the presence of reversible airway obstruction (Brigham & West, 2015; *Global Initiative for Asthma*, 2021). While the CTS and Global Initiative for Asthma (GINA) do not list specific criterion for asthma severity, the National Heart, Lung and Blood Institute (NHLBI) defines mild asthma to include an $FEV_1 > 80\%$ of the predicted value (Yang et al., 2021).

Bronchoprovocation testing utilizes spirometry to monitor induced bronchoconstriction by measuring FEV_1 data. During bronchoprovocation testing, shortened spirometry maneuvers consist of a maximal inspiration to TLC followed by a blast expiration for at least 2-seconds (Coates et al., 2017). Shortened maneuvers reduce fatigue while still producing FEV_1 data, the outcome required to demonstrate a fall in lung function. The quality of bronchoprovocation tests relies on the participant's ability to perform reproducible spirometric maneuvers.

2.1.1 Method

During each study visit, spirometry was used to establish a baseline FEV_1 using an nSpire KoKo® spirometer (Carestream Medical Ltd, Oakville, Ontario, Canada). Baseline lung function was evaluated based on FEV_1 and forced vital capacity (FVC) data using standardized equations (Quanjer et al., 2012).

2.2 Bronchoprovocation Testing

AHR is a key characteristic of asthma and transient airway responses can be induced by direct and indirect acting stimuli (Pawankar et al., 2009). Direct agonists, including methacholine and histamine, elicit airway narrowing through direct interaction with receptors on airway smooth muscle cells. Indirect stimuli such as exercise, adenosine monophosphate, mannitol and allergen, act to elicit airway narrowing via indirect pathways resulting in the release of endogenous bronchoconstricting stimuli from surrounding cells (e.g. histamine from mast cells) as well as pro-inflammatory cytokines (Coates et al., 2017). Bronchoprovocation testing with direct (e.g. methacholine) and indirect-acting stimuli (e.g. allergen) is an invaluable tool used in clinical asthma research. Early investigations with histamine in the 1940s demonstrated bronchoconstriction occurring at lower doses and to a greater extent in asthmatics, leading to the development of AHR testing with direct stimuli as diagnostic measures (Davis & Cockcroft, 2012). Normal airways do not usually respond significantly to direct acting stimuli within the dose range required to induce bronchoconstriction in hyperresponsive asthmatic airways. Asthma pharmacotherapies often undergo investigations with methacholine challenge testing to effectively measure drug efficacy, duration, tolerance, and safety. For more than three decades, allergen bronchoprovocation testing has been employed to mimic various acute and chronic features of allergic asthma in the laboratory and provides a tool to evaluate asthma therapies (Diamant et al., 2013).

2.2.1 Methacholine Challenge Testing

Methacholine (MCh) is a synthetic structural analog of the neurotransmitter acetylcholine that can directly stimulate M_3 muscarinic receptors on airway smooth muscle, inducing bronchoconstriction in hyperresponsive airways (Coates et al., 2017). While AHR to methacholine is a cardinal feature of asthma, it has been documented in other airway disorders including COPD, cystic fibrosis, and allergic rhinitis (Davis & Cockcroft, 2012). AHR to direct

stimuli (e.g. methacholine) is quantified by the provocative dose (MCh PD_{20}) or concentration (MCh PC_{20}) causing a 20% fall in baseline FEV_1 (Coates et al., 2017; Cockcroft et al., 1983; Davis et al., 2017). AHR to methacholine is characterized by increased sensitivity or lower PD_{20}, and increased reactivity (Davis et al., 2018). Methacholine Challenge Testing (MCT) can be used in research and clinically as a diagnostic agent to confirm the presence of AHR to methacholine that is suggestive of asthma (Davis & Cockcroft, 2012). If MCh $PD_{20} > 400\mu g$, the MCT is considered negative (Coates et al., 2017). The negative predictive power of the MCT can rule out clinically current AHR to methacholine with reasonable certainty. Taken together, a negative MCh PD_{20}, normal lung function parameters (e.g. FEV_1,) and the absence of bronchodilator response can refute an asthma diagnosis. Within a stable mild asthmatic individual, MCT is highly reproducible and MCh PD_{20} tends to vary less than 1 doubling dose (Inman et al., 1998). Therefore, a difference in MCh $PD_{20} \geq 1$ doubling dose is assumed to be clinically significant (Inman & Norman, 2006) and suggests improved (i.e. increased MCh PD_{20}) or worsened (i.e. decreased MCh PD_{20}) control. However, false negative challenges (MCh $PD_{20} > 400\mu g$) can occur in atopic asthmatics outside of allergy season (Davis & Cockcroft, 2012). In addition to assessing the level of AHR in an individual, MCT are also used to evaluate the bronchoprotective effect of pharmacotherapies. Bronchoprotection occurs when a drug antagonizes methacholine-induced bronchoconstriction. Anticholinergic therapies such as ipratropium, are competitive M_3 receptor antagonists which block methacholine binding, thereby preventing bronchoconstriction (Crimi et al., 1992; Davis et al., 2018). However, increasing the dose of methacholine will overcome the inhibitory effect, induce bronchoconstriction, and shift the dose response curve to the left (Blais, Davis, et al., 2017).

2.2.1.1 Method

Methacholine challenges were performed in the current studies (Chapters 3 and 4) according to the volumetric method (Davis et al., 2017). Following baseline spirometry, participants inhaled 0.5mL of aerosolized normal saline (diluent) by tidal breathing, using the Aerogen Solo® vibrating mesh nebulizer (Canadian Hospital Specialties Ltd, Oakville, Ontario, Canada) until the entire volume had been delivered (~ 2-minutes). At 30- and 90-seconds post-inhalation, a shortened spirometry maneuver was performed to obtain FEV_1. Administration of the diluent controlled for any reactivity to the inhalation of a mist prior to inhalation of the constricting

agent, methacholine. A fall in FEV_1 >10% from baseline following diluent indicates sensitivity to the inhalation of a mist and the MCT would be continued with caution. The lowest of 30- and 90-second post-diluent FEV_1 values was used to establish a target fall in FEV_1 of at least 20%.

Provocholine (Methapharm, Inc., Brantford, Ontario, Canada) reconstituted with normal saline was prepared in doubling dilutions from 2.5µg up to 1600µg (Chapter 3) or 400µg (Chapter 4) for methacholine inhalation. Five-minutes after beginning diluent inhalation, the first 0.5mL dose of methacholine was aerosolized to completion, followed again by FEV_1 maneuvers at 30-seconds and 90-seconds. Subsequent doses were administered in a doubling fashion at 5-minute intervals until a fall in $FEV_1 \geq 17\%$ had been achieved or the maximum dose of methacholine had been administered. The maximum dose of methacholine that could be administered varied according to the study protocol and study visit requirements. MCh PD_{20} values were calculated using interpolated (formula 2.1) or extrapolation (formula 2.2), respectively.

Formula 2.1 fall in FEV_1 at final dose > 20% (Cockcroft et al., 1983; Davis et al., 2017):

$$\textit{Interpolated MCh } PD_{20} = antilog\ [log\ D_1 + \frac{[(log\ D_2 - log D_1)(20 - R_1)]}{R_2 - R_1}]$$

D_1 = second to last dose of MCh administered

D_2 = last dose of MCh administered causing a fall in $FEV_1 \geq 20\%$

R_1 = the fall in FEV_1 after D_1

R_2 = the fall in FEV_1 after D_2

Formula 2.2 fall in FEV_1 at final dose ≥ 17% and < 20% (Jokic et al., 1998):

$$\textit{Extrapolated MCh } PD_{20} = [\frac{20}{last\ \%\ fall\ in\ FEV_1}]\ x\ last\ dose\ of\ methacholine\ administered$$

The magnitude of bronchoprotection (i.e. the dose shift) provided by asthma therapies (e.g. bronchodilators) was quantified using **Formula 2.3** (O'Connor et al., 1992):

$$\textit{Doubling dose shift} = \frac{(logPD_{20\ post-dose} - logPD_{20\ baseline})}{0.3}$$

MCT can help predict response to allergen as early airway responses are dependent on the degree of AHR to non-allergic stimuli (e.g. methacholine or histamine) and levels of allergen-specific circulating IgE (Boulet et al., 2007; Cockcroft et al., 2019). The prediction equation (Formula 2.4) was developed using the relationship between the provocative concentration of allergen causing a 20% fall in FEV_1 (allergen PC_{20}) and histamine PC_{20} data (Cockcroft et al., 1987). Historically, histamine PC_{20} has been interchangeable with MCh PC_{20} for investigations using the Wright® nebulizer to deliver MCh (Cockcroft et al., 2005). This equation can be effectively used to predict allergen responsiveness with the Solo® nebulizer by modifying for differences between the nebulizers and using Solo® MCh PD_{20} data (converted to equivalent PC_{20} data) in the prediction equation (Cockcroft et al., 2019). It has been established that a Wright® MCh PC_{20} of 16mg/mL equates to a Solo® PD_{20} of 400µg (Blais et al., 2018; Davis et al., 2017). Therefore, a MCh PD_{20} generated with the Solo® nebulizer can be converted into an equivalent PC_{20} to be used in the allergen prediction equation by using the relationship in **Formula 2.4** (Davis et al., 2017):

$$Converted\ MCh\ PC_{20} = \frac{MCh\ PD_{20}}{25}$$

After sufficient allergen bronchoprovocation testing data with the Solo® nebulizer are obtained, a prediction equation specific to this nebulizer could be developed.

2.2.2 Allergen Inhalation Challenge

Allergen-induced mast cell degranulation of mediators including histamine and CysLTs are responsible for acute bronchoconstriction captured as the fall in FEV_1 during the early asthmatic response (EAR) (Davis et al., 2005; Gauvreau et al., 2015). Histamine and CysLTs also induce bronchoconstriction during the late asthmatic response (LAR) (Davis et al., 2009). The EAR is defined as the maximum fall in FEV_1 that manifests between 0 to 3-hours following allergen inhalation while the LAR is the maximum fall in FEV_1 beginning 3- to 8-hours after allergen (Blais, Cockcroft, et al., 2017). The EAR may also be quantified as the provocative dose (EAR PD_{20}) or concentration (EAR PC_{20}) of allergen required to elicit a 20% fall in FEV_1.

Allergen inhalation challenges (AIC) are useful to look at efficacy of drugs against the allergen-induced asthmatic responses (Inman et al., 1995). When the dose of allergen administered is kept constant within an individual, both the EAR and LAR are reproducible.

Thus, the allergen challenge can be used as a tool when evaluating asthma medications to detect changes in allergen-induced asthmatic responses.

The EAR is dependent on both non-allergic airway responsiveness and the level of allergen specific IgE (Cockcroft et al., 2019). Therefore, MCT, Skin Prick Test (SPT) and Skin Test Endpoint (STE) titration can be used to predict the EAR PC_{20}. Skin tests diagnose allergy by demonstrating IgE mediated allergic sensitivities (Pawankar et al., 2009). There is a high degree of correlation between reported allergic asthma symptoms, skin prick tests and provocative challenges; therefore, skin tests are a first-line diagnostic method prior to allergen inhalation challenges.

In a SPT, both positive and negative controls are used to control for inter-patient variability in cutaneous reactivity (Pawankar et al., 2009). The negative control is important in dermographic patients who display wheal and erythema in response to a prick alone. The negative control can also detect differences in pricking device such as a lancet or syringe as well as variations in tester technique. Variability between tester technique can be controlled for by ensuring that the same individual performs all skin prick tests within a study (Blais et al., 2019). A reaction at the negative control site can impair the interpretation of allergen reaction sites (Pawankar et al., 2009). The positive control site serves as a comparison for the relative sensitivity of allergen sites and can detect rare patients who are poorly reactive to histamine or have accidentally taken an antihistamine medication.

2.2.2.1 Method

In the tiotropium study (Chapter 4), the allergen for the allergen inhalation challenge was selected from available study allergens based on the largest wheal response from the SPT and a clinically relevant history of inducing allergic asthma. Available allergens were obtained from Omega Laboratories, Montreal QC or Hollister Stier, Spokane, Washington and included standardized extracts of cat hair, timothy grass, and house dust mite *dermatophagoides pteronyssinus* (HDM-DP) as well as a negative control and histamine (positive control). Wheal measurements were taken at their peak reaction time between 10-15-minutes by two perpendicular measurements of the wheal in millimetres.

Table 2.1 *Stock concentrations for standardized allergen extracts.*

Allergen	Cat hair	Timothy grass	HDM-DP
Concentration (units/mL)	10,000	100,000	30,000

Abbreviations: HDM-DP: house-dust mite DP.

Allergens were initially diluted to 1:8 and subsequent 2-fold dilutions were performed to create the range of concentrations from 1:8 to 1:32,000. The STE was determined as previously described (Blais et al., 2019) using duplicate skin tests with the mean wheal size measured at 10-minutes. The weakest concentration of the selected allergen that induced a 2-mm wheal or less was established as the STE. Once the wheal response was recorded, topical corticosteroid cream and/or an ice pack was provided as necessary.

In combination with the MCh PC_{20}, the STE was used to predict the EAR PC_{20} using **Formula 2.5** (Cockcroft et al., 1987):

$$Predicted\ EAR\ PC_{20} = 0.68\ x\ log(MCh\ PC_{20}\ x\ STE\ Dilution)$$

To account for the differences in the predicted EAR PC_{20} in terms of the Wright® nebulizer and the EAR PD_{20} with the Solo® nebulizer, the starting dilution for the allergen challenge was set at 6-doubling dilutions below the predicted EAR PC_{20}. Prior to the allergen challenge, spirometry was measured in triplicate with the highest FEV_1 value providing the baseline to which a 20% fall in FEV_1 post-allergen inhalation would be determined (Cockcroft et al., 2019). Doubling 0.5mL doses of allergen were inhaled to completion by tidal breathing at 12-minute intervals using the Solo® nebulizer. Duplicate (one-minute apart) FEV_1 measurements were performed 10-minutes after allergen inhalation. If the FEV_1 fell 16-19.9%, a spirometry maneuver was repeated after an additional 10-minutes and if lung function improved, another dose of allergen was administered. If after 10-minutes the FEV_1 further declined or stayed the same, no further doses were given. The response was followed at timed intervals up until 5-hours post-inhalation. After collecting data on inflammatory measures, a 200µg dose of salbutamol was inhaled to reverse any lasting bronchoconstriction. To prevent the development of a late response, a single 500µg dose of fluticasone propionate was inhaled. Participants were allowed to leave the laboratory when the FEV_1 was at least 90% of baseline.

The delivered dose of allergen was calculated by **Formula 2.6** (Cockcroft et al., 2019) using allergen concentrations from Table 2.1:

$$\textit{Delivered Dose of Allergen} = \textit{Allergen } (\frac{units}{mL}) \textit{ x Nebulizer volume x Respiratory duty cycle}$$

Solo® Nebulizer volume = 0.5mL

Respiratory duty cycle = 0.4 (Blais et al., 2020; Coates et al., 2017)

Allergen administration via the Solo® nebulizer using the volumetric method produced data to calculate the EAR PD_{20}. If the maximum fall in FEV_1 was ≥ 20%, **Formula 2.7** was used to interpolate EAR PD_{20} (Cockcroft et al., 1983):

$$\textit{Interpolated EAR } PD_{20} = antilog\ [log\ A_1 + \frac{[(log\ A_2 - log A_1\ )(20 - R_1)]}{R_2 - R_1}]$$

A_1 = second to last dose of allergen administered

A_2 = last dose of allergen administered causing a fall in FEV_1 ≥ 20%

R_1 = the fall in FEV_1 after A_1

R_2 = the maximum fall in FEV_1 after A_2 occurring 10-minutes to 1-hour after inhalation

If the maximum fall in FEV_1 was < 20%, **Formula 2.8** was used to extrapolate the EAR PD_{20} (Jokic et al., 1998):

$$\textit{Extrapolated EAR } PD_{20} = [\frac{20}{maximum\ \%\ fall\ in\ FEV_1\ after\ last\ dose}]\ x\ last\ dose\ of\ allergen\ administered$$

2.3 Measures of Inflammation

Airway inflammation is central to asthma, making it an important variable to monitor in trials on asthma management therapies (Yokoyama et al., 2019). Various biomarkers of inflammation can provide non-invasive practical ways of monitoring the level of inflammation of the airways (Pawankar et al., 2009). These indirect measures, including levels of fractional exhaled nitric oxide and sputum differential cell counts, can serve as replacements for the invasive direct sampling measures including bronchoalveolar lavage and biopsy.

2.3.1 Fractional Exhaled Nitric Oxide

Nitric oxide (NO) has an established role in lung biology and the pathophysiology of respiratory disease (Dweik et al., 2011). NO can act as a vasodilator, mild bronchodilator, neurotransmitter and inflammatory mediator in the lungs and airways. The fraction of nitric oxide found in exhaled air (FeNO) can provide a biomarker of lower respiratory tract inflammation and is a beneficial procedure in respiratory research as it allows serial real-time measurements to monitor inflammatory status (Lieberman, 2007; Yokoyama et al., 2019). In uncontrolled asthmatics, high FeNO correlates with airway eosinophilia, a key component of allergic airway inflammation (Pawankar et al., 2009). However, FeNO is not specific for asthma as elevations in FeNO occur in non-asthmatic conditions including eosinophilic bronchitis, atopy, and allergic rhinitis (*Global Initiative for Asthma*, 2021). FeNO is closely associated with eosinophilic inflammation; however, raised FeNO does not accurately predict percentages of sputum eosinophils (Hastie et al., 2013). Therefore, FeNO measurements are a useful clinical tool in the diagnosis and phenotyping of asthma but cannot diagnose allergic asthma and eosinophilic inflammation alone.

Elevated FeNO in allergic asthma is a result of increased production of NO by nitric oxide synthase (NOS) during the conversion of L-arginine into L-citrulline (Yokoyama et al., 2019). In the body, there are three identified isoforms of this enzyme that vary in their expression, localization, and molecular characteristics: neuronal NOS (nNOS), inducible NOS (iNOS) and epithelial NOS (eNOS). While all three isoforms can exist in the airways, nNOS and eNOS are constitutively expressed and activated by agonist-dependent increases in Ca^{2+} in the cytoplasm. Inflammatory cytokines stimulate production of mRNA for the inducible, Ca^{2+}-independent form the enzyme, iNOS. iNOS has a greater capacity for NO production and a longer endurance, supplying a greater proportion of FeNO when activated. The inflammatory cytokines IL-4, IL-5 and IL-13 are produced by activated Th2 cells, ILC2 and mast cells in the airways of asthmatic individuals. While NO is present in the exhaled air of healthy individuals, individuals with airway inflammation due to inflammatory cytokines would have a greater concentration of NO in their exhaled air.

American Thoracic Society (ATS) guidelines define high FeNO indicating eosinophilic inflammation at levels > 50ppb (Dweik et al., 2011). Levels between 25-50ppb are considered

intermediate and should be interpreted with caution. A change in FeNO by 20% in an asthmatic when baseline levels are > 50ppb, or by 10ppb when baseline levels are ≤ 50pb is considered a significant change; however, there are not much data on the clinical significance of FeNO changes (Dweik et al., 2011). Following allergen inhalation, FeNO has been documented to increase by 7-hours and further increase by 24-hours in dual-responders (Kharitonov et al., 1995; Nomani et al., 2016). However, isolated early responders may show no significant increase in FeNO until at least 21-hours (Kharitonov et al., 1995). Immediately after allergen inhalation challenges, FeNO decreases; this is theorized to be caused by the concurrent changes in airway caliber (Haccuria et al., 2014).

2.3.1.1 Method

In the tiotropium allergic asthma study (Chapter 4), FeNO was measured with the NIOX Vero instrument (Circassia AB, UK). Measuring FeNO is reliant on a constant and maintained expiratory flow rate of approximately 50mL/s during the procedure (American Thoracic & European Respiratory, 2005). The flow rate dependence arises from the diffusion of NO from the airway wall into the lumen of the airways. FeNO measurements were comprised of an inhalation to TLC, followed by an exhalation at a flow rate around 50mL/s. The NIOX Vero instrument requires a 10-second-long exhalation to achieve a stable NO plateau from which the result is gathered. Mean plateau concentration over a 3-second window is the reported value in parts per billion (ppb). Two reproducible measurements were taken that did not vary more than 10% and the average was taken for analysis.

2.3.2 Sputum Differential Cell Counts

Eosinophil and neutrophil counts are reproducible biomarkers of airway inflammation. Increases in sputum eosinophils are evident within hours following allergen-induced exacerbations and in individuals with eosinophilic asthma (American Thoracic & European Respiratory, 2005). In healthy non-asthmatics, sputum differential cell counts are predominantly comprised of macrophages (Figure 2.2 Panel C) and neutrophils (Figure 2.2 Panel A), comprising approximately 43% and 50% of the total cell count, respectively (Bacci et al., 2004; Davidson et al., 2013). Sputum eosinophils (Figure 2.2 Panel B) are considered increased when they comprise > 3% of the proportion of cells in the sputum cell count, indicating eosinophilic inflammation. Increases in sputum airway inflammatory cells after allergen inhalation likely

reflects infiltration of the airway walls by these cells (Pin et al., 1992). Cellular infiltration can start as early as 2-hours after allergen and last up to 7-days, evidenced by bronchoalveolar lavage (Lam et al., 1987). Significant eosinophilia in the blood, sputum and bronchial lavage fluid of dual responders following allergen is well documented (De Monchy et al., 1985; Durham & Kay, 1985; Lam et al., 1987; Pin et al., 1992). It is unclear whether this response occurs to the same magnitude in isolated early responders (De Monchy et al., 1985; Durham & Kay, 1985; Pin et al., 1992). Sputum eosinophil counts are a reproducible measurement often used to monitor inflammatory changes after allergen-inhalation (Boulet et al., 2007; Gauvreau et al., 1999). Although sputum eosinophils are often monitored in clinical trials on allergic asthma, airway basophils, mast cells and neutrophils may also increase after allergen (Gauvreau et al., 2000; Imaoka et al., 2011).

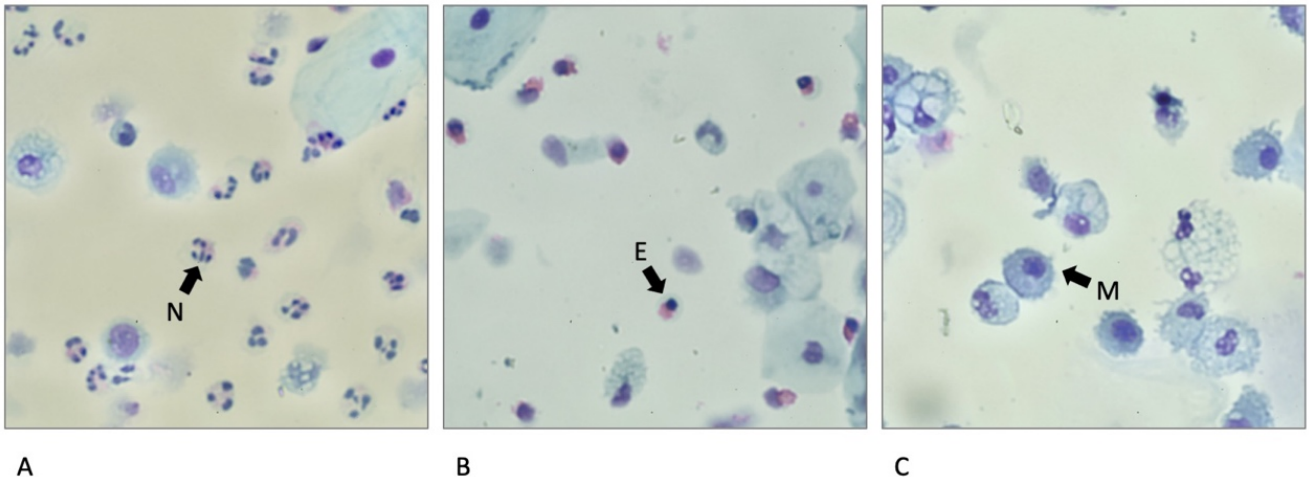

Figure 2.1 *Kwik Diff-stained sputum cells: neutrophils (Panel A), eosinophils (Panel B) and macrophages (Panel C).*

Table 2.2 *Identification characteristics for sputum differential cell counts stained with Kwik Diff.*

Cell Type	Size	Nucleus	Kwik Diff Stain
Eosinophils	9-15uM	2-lobed nucleus	Reddish, pink
Neutrophils	9-15uM	2-5-lobed nucleus	Purple, pink
Lymphocytes	8-16uM	Round nucleus	Pale blue
Macrophage	14-30uM	Multi-nucleated	Blue, purple

Based on descriptions by Pizzichini et al.

2.3.2.1 Method

In the allergic asthma study (Chapter 4), a dose of 200μg salbutamol was administered prior to sputum induction to prevent airway constriction (Pizzichini et al., 1996). An AeroEclipse® II nebulizer (Monaghan Medical, Plattsburgh, New York) was used to deliver hypertonic saline on continuous mode in increasing concentrations each for 7-minutes (7mL of 3%, 5%, and 7%). Following nebulization, participants blew their nose and rinsed out their mouth to avoid contamination from saliva and post-nasal drip. Participants were directed to cough and huff a sample from their chest into a sterile sample cup. At each concentration participants attempted to produce a sample and then performed a shortened spirometry maneuver to monitor for bronchoconstriction. Samples were processed within 2-hours of collection using a slightly modified method (Pizzichini et al., 1996). Mucus plugs were suspended in a volume of cold DPBS equal to 8 times the weight of the sample. The suspension was vortexed, rocked for 15-minutes, vortexed again and then centrifuged (790g) at 4°C for 10 minutes. A volume equal to 4 times the weight of the mucus plugs was drawn off, centrifuged (1500g at 4°C for 10-minutes) and the supernatant aliquoted and frozen for future research. A volume of 0.2% DTT equal to 4 times the weight of the mucus plugs was added to the remaining cell suspension. The sample was then vortexed, rocked for 15-minutes and vortexed again before filtering. Using the tryptan blue exclusion method, total cell count and viability was determined from a 10uL aliquot using a haemocytometer. The sample was then centrifuged at 500g for 10-minutes, the supernatant was drawn-off and frozen for future research. The remaining cell pellet was resuspended in PBS to a cell concentration of $1.0x10^6$ cells/mL. Duplicate cytospins were prepared using 40uL aliquots. The slides were stained with Kwik Diff (Thermo Scientific) and duplicate sputum differential cell counts (sDCC) were performed in a blinded fashion. Cells were identified based on established (Pizzichini et al., 1996) size, nuclei, and staining properties (Table 2.2).

2.4 Methods Summary

Clinical studies evaluating the safety and efficacy of asthma pharmacotherapies utilize a range of tools and procedures to monitor lung function and inflammatory status. These methods can provide insight into the pharmacodynamics of drugs *in vivo*. In the clinical studies described in Chapters 3 and 4, lung function and airway hyperresponsiveness were measured via spirometry and methacholine challenge testing, respectively. These methods were used in

Chapter 3 to monitor for the development of tolerance to bronchoprotection following regular use of a muscarinic antagonist, and in Chapter 4 they were used to follow baseline lung function to confirm the presence of asthma and assess asthma stability over the course of the study. The study outlined in Chapter 4 employed the allergen-inhalation challenge and measures of airway inflammation (FeNO and sDCC) to assess the efficacy of an inhaled muscarinic antagonist on airway responses to inhaled allergen.

3.0 REGULAR USE EFFECT OF INHALED IPRATROPIUM ON METHACHOLINE RESPONSIVENESS IN WELL-CONTROLLED ASTHMATICS

3.1 Objectives

The purpose of this study was to evaluate whether tolerance to the bronchoprotective effect occurred after regular use of ipratropium bromide. Additionally, this study sought to determine if rebound hyperresponsiveness to methacholine occurred following regular use of ipratropium bromide as found by Newcomb et al. This information could be used to inform clinical use and guide further research into muscarinic antagonist use in asthma.

3.2 Hypothesis

Methacholine-induced bronchoconstriction will decrease following daily use of ipratropium bromide and there will be an increase in methacholine responsiveness following treatment withdrawal.

3.3 Relationship to Thesis

This chapter describes the study evaluating methacholine responsiveness following the regular use of ipratropium bromide. This is an 'Accepted Manuscript' of an article published by Taylor & Francis Group in Canadian Journal of Respiratory, Critical Care, and Sleep Medicine on 25 August 2020, available online: https://doi.org/10.1080/24745332.2020.1799263.

3.4 Abstract

Rationale: Short-acting β_2-agonists provide significant bronchoprotection to inhaled methacholine in individuals with asthma. Regular use of β_2-agonists results in the development of tolerance to the bronchoprotective effect. Relatively little is known regarding the development of tolerance following regular use of short-acting muscarinic antagonists.

Objectives: The current study was undertaken to assess bronchoprotection after regular use of inhaled ipratropium bromide ("ipratropium") and after treatment withdrawal.

Methods: Twelve adult participants with mild asthma were assigned to treatment in this double-blind randomized placebo-controlled crossover study. Ipratropium, 40μg thrice daily, and matching placebo were administered for 1 week (22 doses). Methacholine challenges were

performed at baseline, 30-minutes post first and last dose and 24-hours after the final dose. A minimum 7-day washout period separated the treatments.

Measurements and Main Results: Significant bronchoprotection, measured as an increase in methacholine PD_{20} of 4.3 doubling doses (95% CI 3.62-5.06), occurred following the first dose of ipratropium. Bronchoprotection increased slightly to 4.5 doubling doses (95% CI 3.80-5.27) following regular use. Dose shifts following placebo were significantly lower at 0.23 (95% CI -0.30-0.75) and 0.11 (95% CI -0.63-0.85) after the first and last dose respectively ($p < 0.001$ versus ipratropium for both). Changes from baseline methacholine PD_{20} 24-hours after treatment withdrawal were not significant between treatments ($p = 0.19$).

Conclusions: Our data provide evidence that regular use of inhaled ipratropium does not result in loss of bronchoprotection or lead to rebound hyperresponsiveness following treatment withdrawal. Muscarinic antagonists appear to have a superior safety profile over β_2-agonist use in the treatment of asthma.

3.5 Introduction

Asthma is a heterogenous respiratory disease characterized in part by airway inflammation and airway hyperresponsiveness to direct acting stimuli (e.g. methacholine). The bronchoconstriction associated with asthma can be treated with various bronchodilators that act on either adrenergic or muscarinic receptors. Most currently used adrenergic bronchodilators selectively bind β_2 receptors of the airways to relax airway smooth muscle and induce bronchodilation. Muscarinic antagonists bind airway smooth muscle M_3 receptors as the predominant mechanism for inhibition of bronchoconstriction. M_3 receptors elicit airway smooth muscle contraction when stimulated by endogenous acetylcholine or inhaled MCh. MCh is a structural analog of ACh commonly used to induce bronchoconstriction in research and clinical settings. Muscarinic antagonists and β_2-agonists elicit a bronchoprotective effect against MCh-induced constriction via competitive or functional antagonism, respectively.

Tolerance to the bronchoprotective effect following regular use of inhaled β_2-agonists, including both the short-acting agonist salbutamol and the long-acting agonist, salmeterol has been well documented, (Bhagat et al., 1996; Boulet et al., 1998; Cheung et al., 1992; O'Connor et al., 1992) and occurs quite rapidly (Bhagat et al., 1995; Drotar et al., 1998; Stewart et al., 2012).

A decrease in the number of plasma membrane β_2 receptors or "receptor downregulation" has been shown to play a role (Johnson, 2006). Tolerance to the vasoactive properties of various α- and β-adrenergic receptor antagonists via receptor upregulation, or an increase in receptor number following prolonged use, is common in the treatment of cardiovascular disorders (Aarons & Molinoff, 1982; Glaubiger & Lefkowitz, 1977; Vincent et al., 1992). Little is known, however, regarding the development of tolerance to inhaled muscarinic antagonists but receptor upregulation has been observed in other tissues, under different conditions (Emmelin, 1961; Friedman et al., 1983; Lee et al., 1975; Newcomb et al., 1985). The data showing changes in receptor densities following regular use of other drugs targeting G-protein coupled receptors together with evidence that supports muscarinic receptor upregulation suggest the possibility of receptor upregulation and subsequent loss of bronchoprotection following regular use of inhaled ipratropium. Despite the supportive data, a previous study in individuals with asthma failed to elicit a tolerance response to the bronchoprotective effect following regular high dose use of inhaled ipratropium but did document a transient rebound hyperresponsiveness to methacholine 24-hours after treatment withdrawal (Newcomb et al., 1985). The outcomes of the previous study may have been influenced by study design. We therefore undertook the current investigation using standardized methodology and clinically indicated doses to assess the effect of regular use of inhaled ipratropium on both bronchoprotection and rebound hyperresponsiveness to methacholine in individuals with well-controlled asthma.

3.6 Methods

3.6.1 Participants

Twelve non-smoking participants at least 18 years of age with well-controlled mild asthma were recruited. Participants were required to have stable lung function evaluated based on FEV_1 ≥ 65% of predicted and baseline methacholine $PD_{20} \leq 200\mu g$. Participants were allowed to use salbutamol as a rescue bronchodilator, provided it was not used within 6-hours of a study visit. Daily inhaled corticosteroid use was also allowed if dosing was consistent and stable for at least 4-weeks (n = 1). Individuals were ineligible if they had used a long-acting β-agonist or a muscarinic antagonist in the 30-days prior to the study. All other asthma controller or bronchodilator treatments were not allowed. Individuals also had to be devoid of any upper or lower respiratory tract infection for at least 4-weeks prior to study entry. In addition, individuals

were ineligible if they had been exposed to an allergen in the 4-weeks prior to the study, were pregnant or lactating, had diagnoses of diabetes or glaucoma or if they had cardiovascular, urinary, prostate or kidney problems. Written informed consent was obtained prior to performing any study related procedures. The study was approved by the University of Saskatchewan Biomedical Research Ethics Board and registered with clinicaltrials.gov as NCT#04167280.

3.6.2 Methacholine Challenge Test

The MCT, which includes spirometry, was performed as previously described (Chapter 2).

3.6.3 Study Design

The study followed a double-blind, randomized, placebo-controlled, crossover design. In randomized order, participants underwent a treatment period with the study drug, ipratropium, and a treatment period with a matching placebo. Treatment periods were separated by a minimum 7-day washout. During each treatment period, participants were required to take two puffs from the inhaler three times daily for seven days. The first dose and a final dose (dose #22) on Day 8 were administered in the laboratory. Four MCTs were performed during each treatment period; two on Day 1 (single dose effect), one on Day 8 (regular use effect) and one on Day 9 (treatment withdrawal effect). The first MCT was used to determine eligibility and establish a baseline for the remainder of the treatment period. A 30-minute recovery period followed the first MCT prior to administration of the first dose. The second and third MCT's commenced 30-minutes after dosing. The final MCT was completed 24-hours after the final dose. All MCT were completed at the same time of day ± one hour.

Table 3.1 *Visit schedule.*

Visit 1 3-hours	Treatment Period	Visit 2 1-hour	Visit 3 30-minutes	Washout	Visit 4 3-hours	Treatment Period	Visit 5 1-hour	Visit 6 30-minutes
Spirometry Baseline MCT TX #1 Dose 1 Spirometry Post-single dose MCT	Self administer TX #1 for 6-days	Spirometry TX #1 Final Dose Post-regular dosing MCT	Spirometry 24-hour Tx Withdrawal MCT	Minimum 7 days	Spirometry Baseline MCT TX #2 Dose 1 Spirometry Post-single dose MCT	Self administer TX #2 for 6-days	Spirometry TX #2 Final Dose Post-regular dosing MCT	Spirometry 24-hour Tx Withdrawal MCT

Abbreviations: MCT: methacholine challenge test; TX: treatment.

3.6.4 Study Drug and Blinding

Kits comprised of one ipratropium inhaler (Atrovent®) and one placebo inhaler, both pressurized metered dose inhalers, were prepared by one of the investigators not involved in data collection. One of the two canisters was labelled as treatment 1 and the other as treatment 2. A randomization code was sealed in an envelope. Participant 1 received kit 1, treatment 1 first and treatment 2 second. Subsequent participants received subsequent kits in numerical order, each receiving treatment 1 first and treatment 2 second.

3.6.5 Data Analysis

Methacholine PD_{20} data were log transformed prior to analyses. Treatment effect was determined by calculating the dose shift in methacholine PD_{20} and reported as doubling doses. The following formula was used: dose shift = $[(\log PD_{20\text{post-dose}} - \log PD_{20\text{baseline}})/0.3]$ (O'Connor et al., 1992). Between treatment dose shifts and within treatment PD_{20} differences were compared using Student's paired t-test (alpha 0.05) and Statistix 9 software (Analytical software, Tallahassee, Florida). A sample size of twelve participants provided a study power of >90% to detect a one dose difference in methacholine PD_{20}.

3.7 Results

3.7.1 Participants

A total of 22 participants were enrolled in the study, 10 of which failed to meet methacholine PD_{20} inclusion criteria (i.e. had baseline methacholine $PD_{20} \leq 200\mu g$). One participant with a baseline PD_{20} of 228.6μg was included in the investigation based on historical data (i.e. methacholine PD_{20} recently < 200μg) and on level of commitment to participating in a clinical trial (i.e. known to be reliable). Eleven participants followed the study to completion and one additional participant, the only participant using regular ICS (budesonide 200μg bid), was unable to finish treatment 2 (placebo) due to safety measures taken during the COVID-19 pandemic. (Table 1). No unexpected or serious adverse effects occurred. Mean baseline lung function and geometric mean methacholine PD_{20} data were similar for both treatments (Table 1).

Table 3.2 *Participant baseline demographics.*

P #	Sex	Age	Height (cm)	Weight (kg)	Placebo FEV_1 (% predicted)	IB FEV_1 (% predicted)	Placebo Baseline MCh PD_{20}	IB Baseline MCh PD_{20}
1	M	21	177.8	68.2	3.41 (78)	3.6 (83)	154.2	228.6
2	M	43	177.8	88.6	3.49 (84)	3.6 (87)	33.8	76.3
3	M	19	177.8	90.9	3.92 (91)	3.51 (81)	155	146.2
4	F	18	155	63.6	2.65 (99)	2.65(100)	149.5	102.6
5	M	73	167.6	65.9	2.02 (75)	2.03 (75)	28.9	35.9
6	M	35	195.6	84.5	4.95 (92)	4.93 (92)	56.5	38.4
7	F	23	162.1	65.9	3.45 (105)	3.66 (111)	10.0	15.3
8	M	18	177	59.1	4.21 (92)	4.2 (92)	196.4	74.9
9	M	22	166.9	68.2	3.59 (97)	3.57 (96)	89.7	100.0
10	M	29	172.7	79.5	3.86 (91)	4.1 (97)	63.6	136.2
11	M	28	175	70.5	3.42 (78)	3.38 (77)	155.1	183.9
12	F	19	172.3	71	3.46 (88)	3.49 (89)	69.0	123.0
Mean:	75%	29	172.3	71	3.46 (88)	3.49 (89)	72.4[a]	85.1[a]
	M	[16.3]	[10.5]	[10.8]	[0.78]	[0.78]	42-129[b]	51-138[b]

Abbreviations: P#: participant number; FEV_1: forced expiratory volume in 1 second; IB: ipratropium; MCh PD_{20}: dose of methacholine causing a 20% fall in FEV_1; [SD]; [a] Geometric mean; [b] 95% confidence interval.

3.7.2 Bronchoprotection, Tolerance and Rebound Hyperresponsiveness

On average, single dose ipratropium (40μg) significantly decreased airway responsiveness to inhaled methacholine by 4.3 doubling doses (i.e. increased methacholine PD_{20} by more than 16-fold) versus a doubling dose shift of 0.23 following placebo ($p < 0.001$) (Fig. 3.1). After seven days of regular use the magnitude of bronchoprotection increased slightly to 4.5 doubling doses following ipratropium and decreased slightly to 0.11 doubling doses following placebo ($p < 0.001$ for ipratropium versus placebo) (Fig. 3.1). No rebound hyperresponsiveness was detected as dose shifts from baseline to 24-hours were -0.15 and 0.34 doubling doses following ipratropium and placebo withdrawal, respectively ($p = 0.19$) (Fig. 3.1). Individual methacholine PD_{20} at all time points for both treatments are shown in Figure 3.2. Geometric mean PD_{20} data are tabulated in Table 3.2.

Table 3.3 *Geometric mean methacholine PD_{20} (μg) data.*[a]

	Baseline	Single Dose	Regular Dosing	24-hour Post Tx
Ipratropium	85 (51-138)	1698 (813-3467)	1905 (871-4266)	76 (37-155)
Placebo	72 (42-129)	85 (47-155)	79 (35-182)	93 (42-209)

(95% CI); [a] divide by 25 to estimate equivalent Wright 2-minute tidal breathing PC_{20} value.

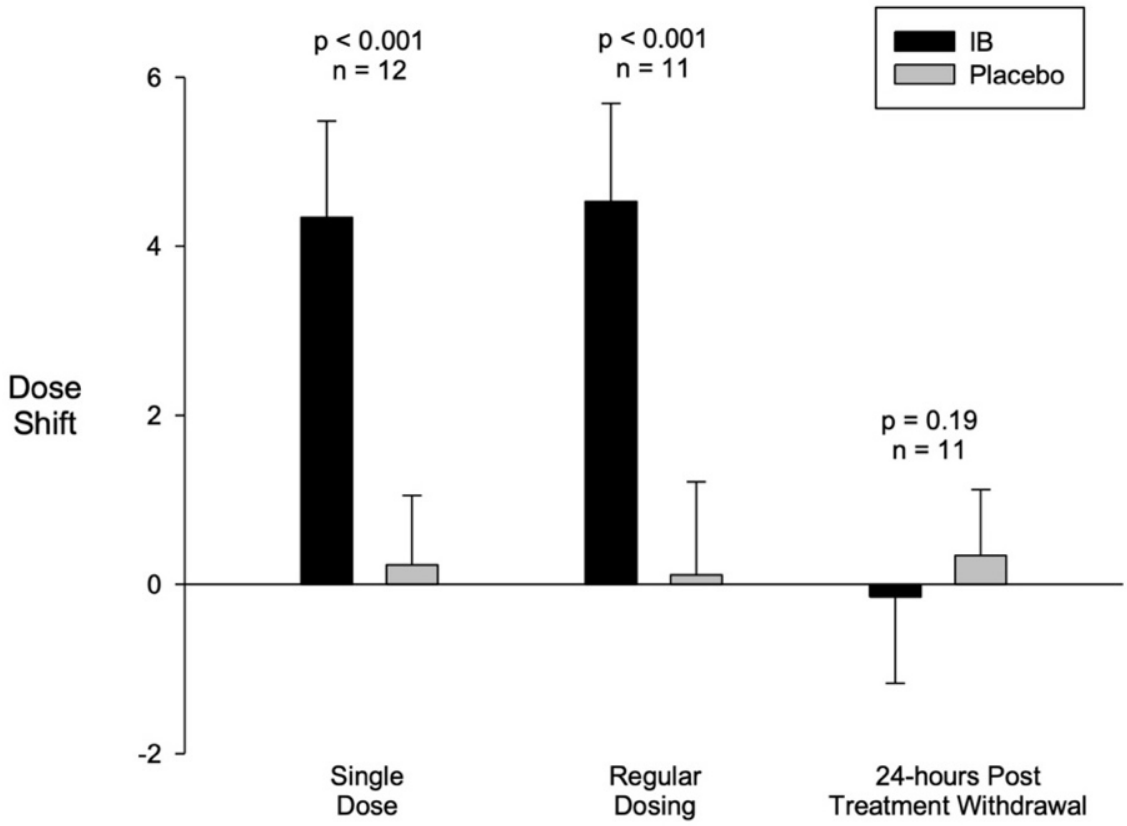

Figure 3.1 *Dose shift (doubling dose) comparisons between ipratropium (IB) and placebo treatments after a single dose (40μg), regular dosing (40μg thrice daily for 7 days) and 24-hours after treatment withdrawal. IB data is a full data set (n=12); Placebo data set is n=12 for single dose and n=11 for regular dosing and 24-hour post treatment withdrawal. Sample size noted on graph relates to statistical analyses.*

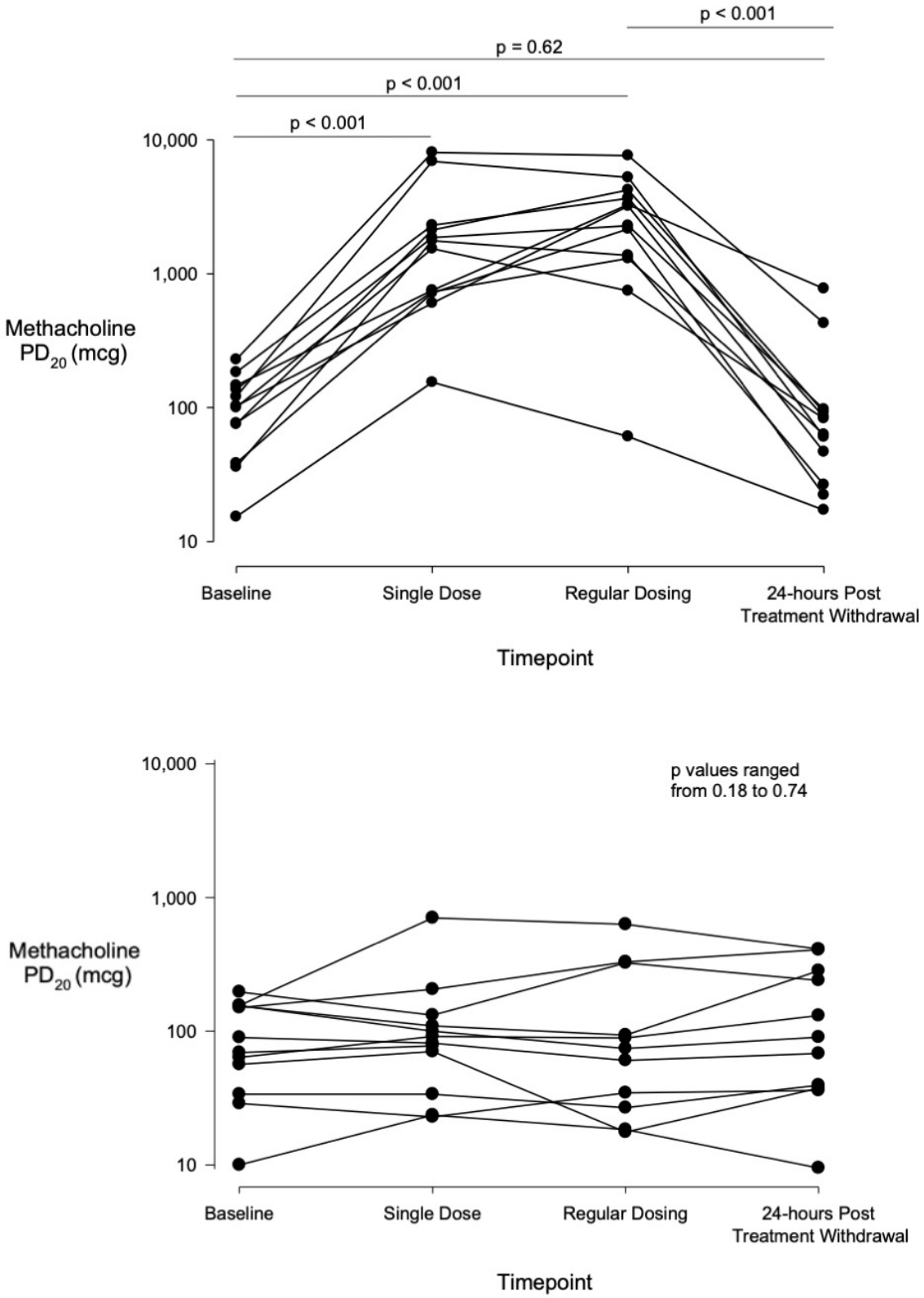

Figure 3.2 *Individual log methacholine PD_{20} data after single dose (2 puffs), regular dosing (2 puffs thrice daily) and following treatment withdrawal (24-hours) for ipratropium (n=12, top) and placebo (n=11, bottom) treatments.*

3.8 Discussion

In our study of twelve mild well-controlled asthmatics, regular use of ipratropium did not result in tolerance to bronchoprotection or rebound hyperresponsiveness following treatment withdrawal. The literature in this area is sparse. We are aware of only one publication from 35-years ago in which tolerance to methacholine induced bronchoconstriction and rebound hyperresponsiveness following regular use of ipratropium were studied (Newcomb et al., 1985). In this previous investigation, tolerance did not develop following regular use of high dose ipratropium (60μg four times daily for 3 weeks), however, transient rebound hyperresponsiveness was documented 24-hours after treatment withdrawal. A major limitation of the previous study was that the highest concentration of MCh administered was only 25mg/mL which was not high enough to elicit a 20% fall in FEV_1 and generate a dose response following treatment. Failure to quantify maximal bronchoprotection to MCh may have skewed the results and underestimated the loss of bronchoprotection following regular use. Additional limitations of the previous study, include the absence of a placebo arm (possible bias due to not accounting for a placebo effect), the small sample size (potentially underpowered with only nine participants) and the use of dosimeter deep inhalation methodology (direct comparison with our current data may not be applicable due to different testing methodologies). Nonetheless, the findings of the previous study (3 puffs four times a day for three weeks; high dose) and our current study (2 puffs three times a day for one week; low dose) suggest regular use of ipratropium does not result in loss of bronchoprotection to methacholine. Based on our knowledge of dose response and the development of tolerance following regular use of β_2 agonists that is dose independent (Bhagat et al., 1996) and rapid onset (Bhagat et al., 1995; Drotar et al., 1998; Stewart et al., 2012) we can infer a similar pattern may occur with muscarinic antagonists. These data suggest that increasing the dose or the duration of dosing with ipratropium are unlikely to result in the development of tolerance. This should be confirmed by prospective investigation.

The absence of tolerance following regular use ipratropium is in stark contrast to the loss of bronchoprotection observed following regular use of both short and long acting β_2-agonists. An early investigation by O'Connor et al showed a small but significant reduction in bronchoprotection against methacholine following 7-days of high dose terbutaline (O'Connor et al., 1992). Bhagat et al showed close to one doubling dose increase in methacholine responsiveness across all doses of salbutamol (200, 400 and 800μg) after one week of daily

treatment (Bhagat et al., 1996). Tolerance has also been shown following 8-weeks (Cheung et al., 1992) and 4 weeks (Boulet et al., 1998) of twice daily salmeterol. Subsequent investigations documented the reduced efficacy to β_2-agonist bronchoprotection to occur quite rapidly. In the case of salmeterol, statistically significant decreases of 44% and 37% in geometric mean methacholine PC_{20} occurred after just two 50μg doses (Bhagat et al., 1995; Drotar et al., 1998); with twice daily salbutamol (200μg q12h) loss of bronchoprotection was observed after just 7 doses (i.e. 3.5 days) (Stewart et al., 2012). As a side note, the addition of inhaled corticosteroids (ICS) as a preventative measure against the loss of bronchoprotection proved ineffective following salmeterol use (Boulet et al., 1998; Kalra et al., 1996) but beneficial, *ex vivo*, following salbutamol use (Cooper & Panettieri, 2008). Recent clinical trial data obtained from a larger sample challenges the previous negative findings not only with respect to the beneficial effects of concomitant ICS but also to the prevalence of tolerance and the role of β_2 receptor downregulation as a potential mechanism (Cardet et al., 2019). One participant in our study was using ICS and this individual produced data consistent with that of the study cohort (i.e. significant single dose bronchoprotection that was sustained after regular use and a slight increase in response 24-hours after treatment withdrawal) suggesting that concomitant ICS use would be unlikely, and not expected, to alter our observed study outcome.

Our findings are inconsistent with the Newcomb study data in that we did not observe rebound hyperresponsiveness to methacholine following treatment withdrawal. A possible explanation for the contradictory findings between our current study and the previous study may be attributed to differences in dosing as a higher dose for a longer duration (60μg given four times daily for 3-weeks compared to our study design of 40μg given three times daily for 1-week) may have led to a state of supersensitivity following treatment withdrawal.

The absence of the development of tolerance in our study suggests muscarinic receptor upregulation did not occur following one week administration of clinically relevant dosing of ipratropium. Given that receptor upregulation is a well-documented phenomenon following prolonged antagonism such as occurs after chronic use of adrenergic receptor antagonists (Aarons & Molinoff, 1982; Glaubiger & Lefkowitz, 1977; Vincent et al., 1992) and with evidence that muscarinic receptor upregulation occurs in non-lung tissue (Emmelin, 1961; Friedman et al., 1983; Lee et al., 1975); and given that receptor downregulation occurs both

quickly and at clinically relevant doses with β_2-agonists within the lung, it is unclear why loss of bronchoprotection to methacholine was not observed in our investigation. One possible explanation could be poor dosing compliance as ipratropium was self-administered for doses 2 through 21. However, we verbally confirmed dosing compliance with participants following regular dosing (i.e., in the laboratory prior to administration of the final dose) and no participant reported any missed doses. Another possible and important consideration for a negative study is that of sample size or study power. We can rule this out as well as a sample size of 12 individuals provides a study power of 90% to detect a one dose difference in methacholine PD_{20} (Inman et al., 1998).

Our findings are limited to clinical data and any mechanistic effects can only be inferred. Evidence has shown that receptor antagonism with some β-blockers does not lead to receptor upregulation and can lead to a decrease in receptor density (Brodde et al., 1986). Another relatively new phenomenon is that of biased antagonism, where the receptor is blocked in a competitive manner but intracellular signalling pathways responsible for regulating receptor numbers (i.e. β arrestin activity) are not affected (Hitchinson et al., 2018). Given the promising data that biased antagonists have produced, one wonders if a similar strategy (i.e. biased agonism) toward β_2 receptors in the lung would prove beneficial in preventing tolerance but maintaining the excellent bronchoprotective and bronchodilatory properties. While these mechanistic data are intriguing, the relevance to explaining the possible mechanistic rationale for the absence of tolerance following ipratropium use is limited (i.e. intrinsic sympathomimetic activity and small peptide molecules). *Ex vivo* muscarinic M_3 receptor binding assays using human lung tissue would be one method for determining whether receptor upregulation occurs on airway smooth muscle following prolonged ipratropium use. Consideration might also be given to the non-selective receptor binding properties of ipratropium and the potential role concomitant antagonism of muscarinic subtypes 1 and 2 may play in regulating M_3 receptor numbers. Given the class effect on the development of tolerance following regular use of β_2 agonists (i.e. loss of bronchoprotection occurs with both short and long-acting β-agonists) a clinical investigation on loss of bronchoprotection following regular use of tiotropium may provide additional insight. Although anticipated to also be a negative study based on β_2 agonist data, prolonged receptor occupancy and receptor specificity for the M_3 subtype are rationale supporting a different outcome.

With respect to asthma, ipratropium is only indicated for acute use in the management of exacerbations, therefore, potential clinical implications from our findings relate to off label use in the routine management of asthma and, indirectly, to use in COPD where treatment with anticholinergics is both recommended and commonly used. In light of the well-documented detrimental effects of regular β_2-agonist use in the treatment of asthma, it may be useful to further investigate, or perhaps renew our interest in the mechanisms and clinical effects of targeting the cholinergic system and the use of muscarinic antagonists as alternative treatments.

4.0 TIOTROPIUM EFFICACY AGAINST ALLERGEN INDUCED EARLY ASTHMATIC RESPONSES

4.1 Objective

The primary objective was to determine if regular tiotropium affects allergen-induced early asthmatic responses. Our secondary objective was to use indirect measures of airway inflammation to assess if tiotropium influences airway inflammation (i.e. Th2 phenotype).

4.2 Hypothesis

Tiotropium will attenuate allergen induced airway responses by blocking M_3 receptors and disrupting ACh-TSLP synergism that is promoting the Th2 phenotype in allergic asthma.

4.3 Relationship to Thesis

This chapter describes the study on allergen responsiveness and inflammation following regular tiotropium. This manuscript will be submitted for publication.

4.4 Abstract

Rationale: Acetylcholine promotes an allergic or type 2 response via muscarinic receptors on dendritic cells. Single dose SAMA show equivocal results on allergen-induced asthmatic responses. The effect of LAMA, such as tiotropium bromide monohydrate (Spiriva® Respimat®), on allergen-induced asthmatic responses is unknown.

Objectives: The study aimed to assess the efficacy of regular use tiotropium against allergen-induced early asthmatic responses and airway inflammation.

Methods: Thirteen adult participants with mild allergic asthma were assigned to treatment in this double-blind, randomized, placebo-controlled crossover study. Tiotropium, 5µg once daily, and matching placebo were each administered for 7-days. Allergen inhalation challenges were performed 30-minutes following the final dose on Day 8. Measures of airway inflammation (FeNO and sDCC) were collected at baseline, before allergen inhalation (FeNO only), and 5-hours after allergen inhalation. A minimum 14-day washout separated the treatment periods.

Measurements and Main Results: Regular use of tiotropium worsened the early asthmatic response to allergen as evidenced by a decrease in EAR PD_{20} from a geometric mean of 22.2 allergen units (SE = 0.14) following placebo treatment to 16.7 allergen units following

tiotropium treatment (p = 0.054). No significant difference in the level of FeNO was observed following tiotropium treatment. The allergen induced increase in sputum eosinophils was also unchanged following tiotropium treatment; sputum eosinophils increased by 14.5% and 15.2% for tiotropium and placebo treatments, respectively (p = 0.91)

Conclusions: Our data suggest regular use of tiotropium does not alter airway responses to inhaled allergen in mild atopic asthmatics. A trend toward a worsening of the early asthmatic response was observed although this did not coincide with a trend toward worsened airway inflammation. Data surrounding the efficacy of muscarinic antagonists on allergen induced responses remains inconclusive.

4.5 Introduction

There are accumulating data on the role of non-neuronal cholinergic signalling in allergic airway responses (Gori et al., 2019; Gori et al., 2017; Kawashima, 2004; Koyama et al., 1998; Masini et al., 1985; Reinheimer et al., 1997). Signalling at non-neuronal muscarinic receptors seems to mediate a proinflammatory signal (Gori et al., 2019). Gori et al demonstrated ACh polarizes dendritic cells towards a type-2 promoting profile via the M_3 muscarinic receptor (Gori et al., 2019; Gori et al., 2017). Therefore, inhaled muscarinic antagonists may protect the airways by inhibiting ACh-induced polarization. Data from ovalbumin challenged animals treated with muscarinic antagonists show reductions in allergen-induced changes (Bos et al., 2007; Ohta et al., 2010). In a recent *ex vivo* dendritic cell investigation, synergism between ACh and the epithelial cytokine, TSLP, in promoting type 2 responses was shown and effectively inhibited by muscarinic antagonism (Gori et al., 2017). Data documenting anti-TSLP efficacy in the human allergen challenge model shows a significant but not complete reduction in allergen-induced changes (Gauvreau, Hohlfeld, et al., 2020; Gauvreau et al., 2014). The residual response could be due to intact cholinergic signalling on dendritic cell muscarinic receptors. However, pro-inflammatory cholinergic signalling is not conserved amongst all cells involved in immune responses. By contrast, ACh appears to modulate mast cell degranulation via the M_1 muscarinic receptor, partially inhibiting the release of bronchoconstricting mediators (e.g. histamine and cysteinyl leukotrienes) (Reinheimer et al., 1997; Reinheimer et al., 2000). Based on these conflicting data it is unknown whether airway muscarinic antagonism would have anti-inflammatory or pro-inflammatory effects on allergen-induced airway responses. While SAMA

efficacy against allergen-induced constriction has been evaluated (Clarke et al., 1982; Cockcroft et al., 1978; Fish et al., 1977; Howarth et al., 1985; Itkin & Anand, 1970; Orehek et al., 1975; Ruffin et al., 1978; Yu et al., 1972), there are no human data on long-acting formulations. Tiotropium bromide (Spiriva® Respimat®) is the only LAMA currently approved in the management of asthma, with affinity for both M_1 and M_3 muscarinic receptors. Collectively, these data suggest that the effects of LAMA on allergen-induced airway responses should be evaluated in the human allergic asthma model.

Asthma is a chronic respiratory disease characterized by reversible airway obstruction, airway inflammation, hyperresponsiveness to stimuli and airway remodelling. A major environmental factor involved in asthma development is allergen exposure. In sensitized individuals, allergen induced cellular responses lead to type 2 inflammation and bronchoconstriction that can manifest shortly after allergen exposure, known as the allergen-induced EAR. A portion of allergic asthmatics may experience recurrence of bronchoconstriction 3-8 hours after allergen, known as the LAR. Individuals who experience both early and late responses to allergen are dual responders. Both early (Davis et al., 2005) and late (Davis et al., 2009) bronchoconstriction responses to allergen are due to release of histamine and CysLTs. Late responses are associated with increased airway inflammation and recruitment of effector cells, most predominantly eosinophils (Gauvreau et al., 2015). The allergen inhalation challenge has become an important tool for studying pharmacological modulation of allergic airway responses. The magnitude of allergen-induced inflammation can be indirectly quantified using measures of FeNO and sDCC of inflammatory cells. Using standardized methodology, we therefore undertook this investigation to assess the effect of regular inhaled tiotropium on both allergen-induced bronchoconstriction during the early asthmatic response and allergen-induced airway inflammation.

4.6 Methods

4.6.1 Participants

Eligible participants had well-controlled mild allergic asthma, were non-smoking and between 18-65 years old. Stable baseline lung function was required for study enrollment and FEV_1 had to be ≥ 80% of predicted. Asthma management had to require less than daily use of short-acting β_2 agonist for occasional symptoms. Individuals were ineligible for study enrollment if they

required current use of regular inhaled corticosteroids, inhaled muscarinic antagonists, combination therapy, biologics, leukotriene receptor antagonists or antihistamines to manage asthma and/or allergy. Participants were required to have a positive response to inhaled MCh with MCh PD_{20} being ≤ 400μg. Atopy was evidenced by a positive skin prick test to at least one of three common allergens and a clinical history of asthma symptom development upon exposure. Participants were required to have not been exposed to allergen in the 4-weeks prior to beginning the study. Participants were required to be free of respiratory infection for 4-weeks prior to study entry. In addition, any recent travel, symptoms of COVID-19 or known exposure to a positive COVID-19 case required a 14-day interval prior to study visits. Individuals were excluded for the following medical conditions: pregnancy, current breast-feeding, narrow angle glaucoma, urinary retention or known hypersensitivity to tiotropium bromide or Spiriva® Respimat® formulation components. Use of non-steroidal anti-inflammatory medications was prohibited for 3-days prior to any study visit, and salbutamol use was prohibited within 6-hours of a study visit. Written informed consent was obtained at study visit 1 prior to any procedures. This study was approved by the University of Saskatchewan Biomedical Research Ethics Board, registered with clinicaltrials.gov as NCT#04648813 and received no objection from Health Canada.

4.6.2 Study Design

This study followed a randomized double-blind placebo-controlled crossover design. Participants were randomly assigned to receive tiotropium or a matching placebo during the first treatment period with a minimum 14-day washout separating the two periods (Table 4.1). At the first study visit, atopy to study allergens was established using a skin prick test and skin titration endpoint. The initial visit for each treatment period consisted of a baseline methacholine challenge to monitor non-specific airway responsiveness, and baseline indirect measurements of inflammation by the fraction of exhaled nitric oxide (FeNO), and sputum differential cell counts (sDCC). The first dose was administered at the end of the initial study visit following an inhalation technique tutorial for the use of a Spiriva® Respimat® device. Participants were instructed to self-administer daily doses (i.e. doses #2-#7) at roughly the same time of day. The final dose (dose #8) was administered in the laboratory following baseline spirometry. Thirty-minutes later, a pre-allergen FeNO measurement was taken, and spirometry was repeated. An allergen inhalation challenge commenced as previously described (Cockcroft et al., 2019). The

FEV_1 recovery following allergen was measured at timed intervals up to 5-hours. After 5-hours, inflammatory measures were re-examined using FeNO and sDCC.

Table 4.1 *Visit schedule.*

Visit 1 3-hours	Treatment Period	Visit 2 7-hours	Washout	Visit 3 2-hours	Treatment Period	Visit 4 7-hours
Consent FeNO Spirometry MCT Sputum Induction SPT STE TX #1 Dose 1	Self administer TX #1 for 6-days	Blood pressure Spirometry FeNO Tx #1 Final Dose Spirometry AIC FEV_1 Recovery 5-hours FeNO Sputum Induction	Minimum 2-weeks	FeNO Spirometry MCT Sputum Induction TX #2 Dose 1	Self administer TX #2 for 6-days	Blood pressure Spirometry FeNO Tx #2 Final Dose Spirometry AIC FEV_1 Recovery 5-hours FeNO Sputum Induction

Abbreviations: FeNO: fractional exhaled nitric oxide; MCT: methacholine challenge test; SPT: skin prick test; STE: skin test endpoint titration; TX: treatment; AIC: allergen inhalation challenge.

4.6.3 Laboratory Procedures

All procedures including spirometry, SPT, STE, MCT, AIC, FeNO, sputum induction and sDCC were performed as previously described (Chapter 2).

4.6.4 Study Drug and Blinding

One investigator not involved in data collection prepared kits of one tiotropium Respimat® inhaler (Spiriva® Respimat®) and one matching placebo Respimat® inhaler. Placebo treatments were provided by Boehringer Ingelheim, and active treatments were sourced from the pharmacy at Saskatoon Royal University Hospital. One inhaler was labelled treatment #1, the other treatment #2 and the randomization code was sealed in an envelope. Participant 1 received kit 1 and subsequent kits were supplied in numerical order. Each participant received treatment #1 first and treatment #2 second.

4.6.5 Data Analysis

EAR PD_{20} and FeNO data were log transformed prior to analyses. Treatment effect on the EAR was determined by differences in placebo EAR PD_{20} and tiotropium EAR PD_{20} Treatment effect on FeNO was compared by the difference in FeNO data at baseline to 1-week of treatment and at pre-allergen (after 1-week of treatment) to 5-hours post-allergen. Treatment effect on the percent of sputum eosinophils was expressed as the difference in percent sputum eosinophils at baseline to 5-hours post-allergen compared for tiotropium versus placebo treatments. Treatment differences were evaluated with Student's paired t-test (alpha 0.05) and Statistix 9 software (Analytical software, Tallahassee, Florida). A sample size of thirteen participants provided a study power of > 90% to detect a 50% difference in EAR PD_{20} (Inman et al., 1995). A post hoc non-parametric sign test was used to confirm or refute the paired t-test. A sample size of 8 participants provided a study power of 85% to detect a 35% attenuation of percent sputum eosinophils (Gauvreau et al., 1999).

4.7 Results

4.7.1 Participants

A total of fifteen participants were enrolled in the study. However, two participants were ineligible to continue the study due to a negative skin prick test (i.e. absence of atopy to available allergens) or due to the absence of an asthmatic response to inhaled allergen at visit 2. One participant with historical allergic data only achieved a fall in FEV_1 of 12.8% following allergen inhalation and no further doses were administered due to strong cough response causing discomfort. Thirteen participants followed the study to completion. No unexpected or serious adverse events occurred. Baseline mean FEV_1 and geometric mean methacholine PD_{20} data were similar for the two treatment periods (Table 4.1).

Table 4.2 *Participant baseline demographics.*

P#	Sex	Age	Height (cm)	Weight (kg)	Placebo FEV_1 (% predicted)	Tiotropium FEV_1 (% predicted)	Placebo Baseline MCh PD_{20}	Tio Baseline MCh PD_{20}	Allergen
1	M	44	177.8	90.7	3.44 (84)	3.49 (85)	83.1	69.8	Cat hair
2	M	29	170.2	79.4	3.95 (97)	3.66 (90)	74.7	111.7	Cat hair
3	M	20	177.8	99.8	3.72 (80)	3.77 (81)	144.0	208.3	Cat hair
4	F	21	165.1	74.8	3.37 (98)	3.56 (101)	1,159.4	503.1	HDM DP
5	M	22	170.2	70.3	3.77 (97)	3.72 (96)	78.9	65.8	Cat hair
6	F	34	160.0	63.5	2.85 (94)	2.86 (94)	467.8	90.3	T grass
7	M	52	193.0	117.9	3.99 (86)	3.70 (80)	41.8	38.0	Cat hair
8	F	25	172.8	63.5	3.43 (92)	3.30 (88)	27.0	8.8	Cat hair
9	F	42	167.6	106.6	3.02 (96)	2.88 (91)	583.4	217.4	Cat hair
10	M	39	173.0	83.9	3.72 (93)	3.67 (92)	791.6	384.1	HDM DP
11	F	25	163.0	49.0	2.84 (86)	2.87 (87)	24.3	17.7	HDM DP
13	F	22	167.6	73.9	3.29 (108)	3.12 (103)	23.4	186.9	Cat hair
14	M	30	168.9	79.8	3.76 (94)	3.27 (82)	118.0	221.7	HDM DP
Mean:	54%	31	171.3	81.0	3.47 (93)	3.37 (90)	122.1[a]	100.0[a]	___
	M	[10.2]	[8.3]	[19.0]	[0.39]	[0.35]	54-278[b]	49-204[b]	

Abbreviations: P#: participant number; FEV_1: forced expiratory volume in 1 second; Tio: tiotropium; MCh PD_{20}: dose of methacholine causing a 20% fall in FEV_1; T Grass: timothy grass; HDM DP: house dust mite *dermatophagoides pteronyssinus*; [SD]; [a] Geometric mean; [b] 95% confidence interval.

4.7.2 Early Asthmatic Response

Contrary to the hypothesis, regular tiotropium trended towards increased allergen responsiveness (i.e. lower PD_{20}) during the early asthmatic response with borderline statistical significance ($p = 0.054$). EAR PD_{20} decreased from a geometric mean of 22.2 allergen units (SE = 0.14) with placebo to 16.7 allergen units with tiotropium (SE = 0.14) (Fig. 4.1). Increased allergen responsiveness occurred in ten of thirteen participants with two participants showing a decrease in EAR PD_{20} greater than 1-doubling dose. The remaining three participants showed increased EAR PD_{20} by less than half a doubling dose with tiotropium.

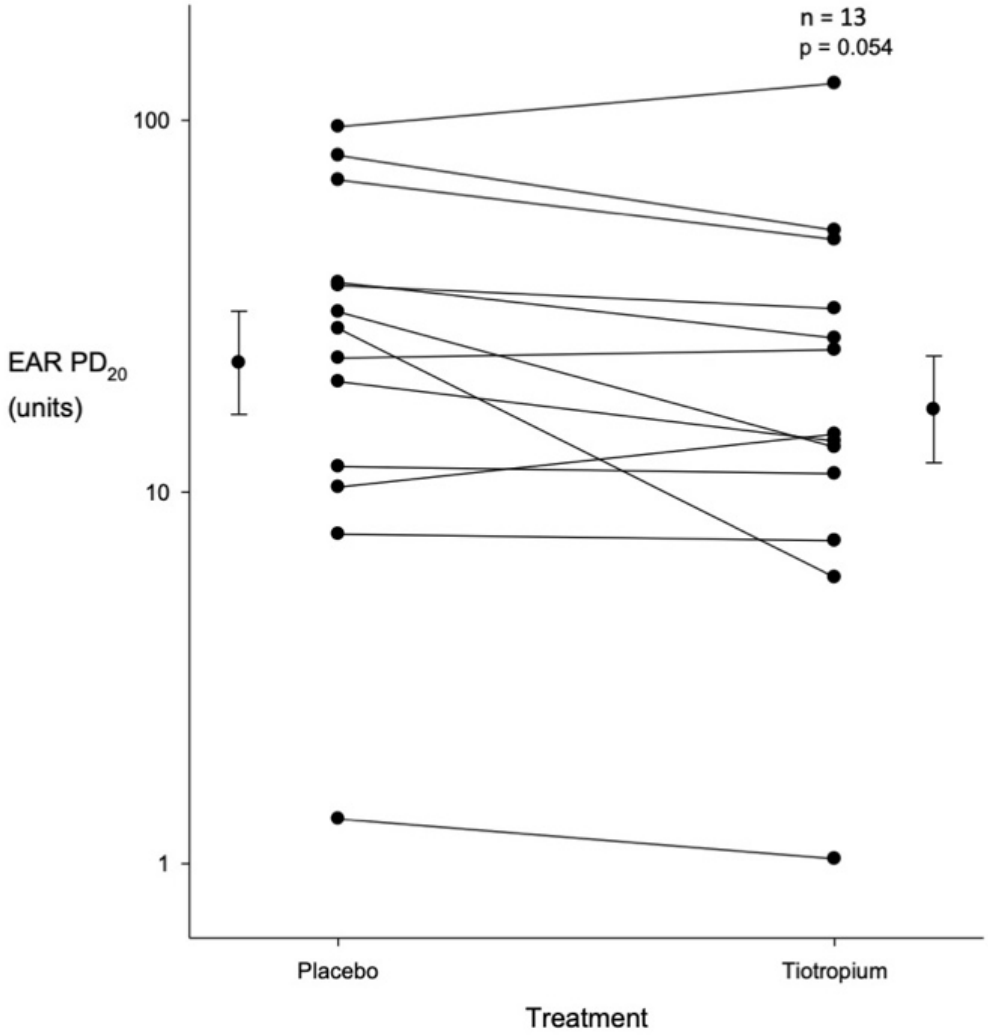

Figure 4.1 *Individual and geometric mean EAR PD_{20} data after placebo and tiotropium (5µg once daily) treatments.*

4.7.3 Inflammatory Measures

The difference in geometric mean FeNO levels did not change from baseline after 1-week of tiotropium treatment compared to placebo (p = 0.29). The difference in FeNO levels from pre-allergen challenge levels (after 1-week of dosing) to 5-hours post allergen challenge also did not significantly differ between treatment periods (p = 0.19). Regular use of tiotropium resulted in an increase in FeNO by 5.4ppb compared to 1.2ppb with placebo treatment (Table 4.3). FeNO levels decreased 5-hours after allergen inhalation in both treatment groups with a mean 2.2ppb decrease following tiotropium and a 4.2ppb decrease following placebo (Table 4.3).

Table 4.3 *Comparison of the geometric mean fractional exhaled nitric oxide (ppb) levels at various timepoints for placebo and tiotropium treatments.*

	Baseline	1-Week of Tx	5-hours Post-Allergen
Tiotropium	30.7 [0.10]	36.1 [0.09]	33.9 [0.08]
Placebo	30.4 [0.09]	31.6 [0.08]	27.4 [0.08]

Abbreviations: [SE].

Eight of thirteen participants were able to produce sputum. In these individuals, the percent of sputum eosinophils increased following allergen inhalation for both placebo and tiotropium treatment (Fig. 4.2) with no significant difference between the two (p = 0.91). Sputum eosinophils increased significantly after allergen inhalation by 14.5% (p = 0.01) and 15.2% (p = 0.002) for tiotropium and placebo treatments, respectively.

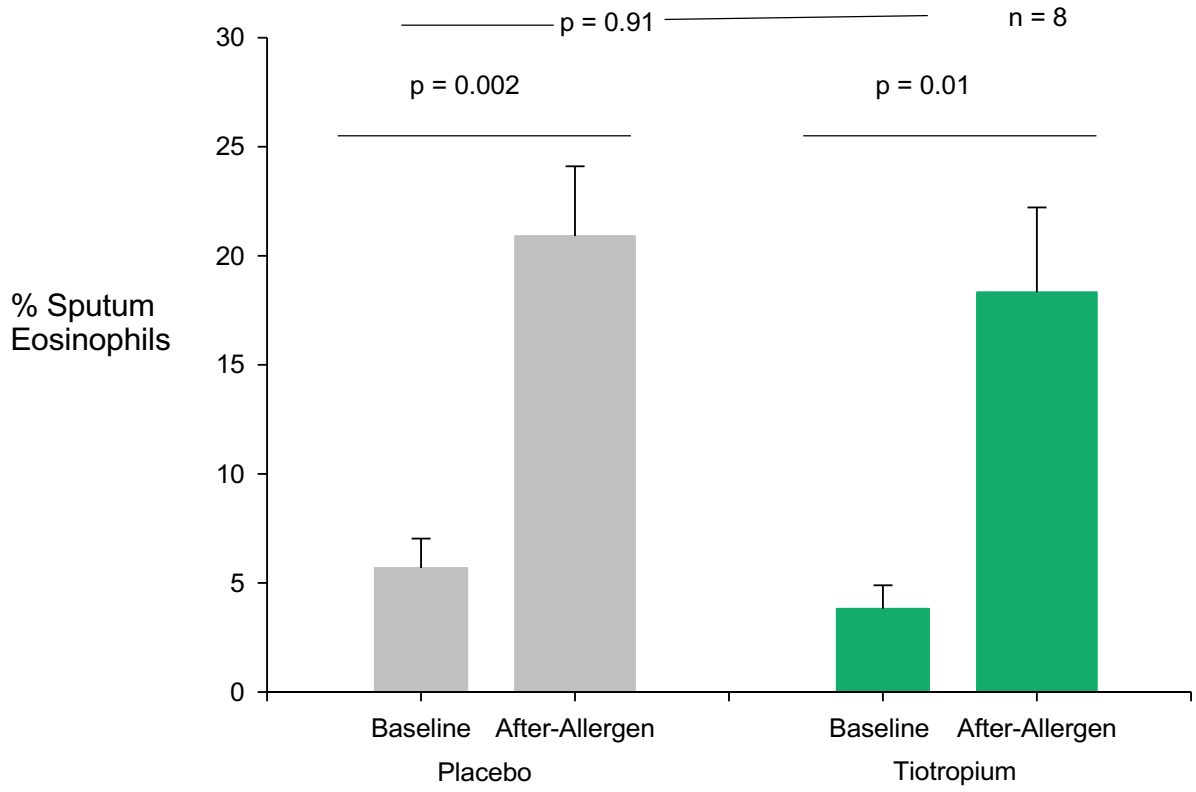

Figure 4.2 *Mean percent of sputum eosinophils in a differential count of 400 cells at baseline and 5-hours after allergen comparisons for placebo and tiotropium treatments.*

4.8 Discussion

In our study of thirteen well-controlled mild allergic asthmatics, daily use of tiotropium led to a non-significant increase in the early asthmatic response to inhaled allergen with no significant effect on levels of FeNO or allergen induced sputum eosinophilia. However, use of Student's paired t-test to evaluate the early asthmatic response assumes that the data are normally distributed. Since EAR PD_{20} for tiotropium versus placebo treatments is nearly statistically significant ($p = 0.054$), and only three of thirteen participants show a different response (slight decrease in allergen responsiveness) it is possible that the data are not normally distributed, and these participants may be outliers. For this reason, we chose to perform a non-parametric analysis of allergen responsiveness (EAR PD_{20}) to confirm or refute the trend observed. A post hoc sign test supported the trend towards increased allergen responsiveness after regular tiotropium with significance ($p = 0.046$).

The effect of single and regular dosing of LAMA on allergen induced asthmatic responses was previously unknown. However, there have been many investigations on single dose SAMA efficacy against allergen induced asthmatic responses. For the most part, these data demonstrate partial efficacy for inhaled SAMA against allergen-induced responses in a portion of allergic asthmatics (Clarke et al., 1982; Cockcroft et al., 1978; Orehek et al., 1975; Ruffin et al., 1978; Yu et al., 1972). However, SAMA shows no attenuation of the early response in another subset of allergic asthmatics (Fish et al., 1977; Howarth et al., 1985; Itkin & Anand, 1970). Therefore, there may be pathophysiologic differences among allergic asthmatics that predisposes some to better protection against allergen with SAMA. The results of this study suggest this efficacy is not conserved with LAMA as regular tiotropium did not attenuate and potentially worsened the early asthmatic response. While a small proportion (3 participants) of the study population showed less response to allergen with tiotropium, the magnitude (< half of a doubling dose) suggests this may be due to natural variation in allergen responsiveness and not a drug effect.

It is unknown whether a single dose of tiotropium would demonstrate increased allergen responsiveness. Data from ovalbumin-challenged animals show promising efficacy of single dose tiotropium against allergen-induced asthmatic responses (EAR and LAR) (Raemdonck et al., 2012; Smit et al., 2014). Clinical investigations with single dose SAMA show no efficacy against allergen-induced LAR (Cockcroft et al., 1978). Single dose tiotropium may also increase

allergen responsiveness, similar to regular tiotropium treatment. However, it cannot be ruled out that regular use of tiotropium may have induced the increased responsiveness to allergen similar to regular β_2-agonist use (Cockcroft et al., 1993; Cockcroft et al., 1995). Salbutamol, dosed 200μg four times daily for 2-weeks significantly increased the EAR by approximately two-fold. At the same dose for 1-week, salbutamol enhanced the LAR causing the maximum fall in FEV_1 to nearly double (Cockcroft et al., 1995). These detrimental effects coincide with tolerance to the bronchoprotective effect of salbutamol against constriction induced by methacholine, adenosine monophosphate and histamine, that occur with both short and long-acting formulations (Jokic et al., 2001; Vathenen et al., 1988). However, single doses of LABA and SABA are effective at inhibiting allergen-induced asthmatic responses (Eiser, 1991; Howarth et al., 1985; Ruffin et al., 1978; Weersink et al., 1994). We recently demonstrated that regular use of ipratropium bromide (SAMA) for 1-week did not produce tolerance to the bronchoprotective effect against methacholine (Cropper et al., 2020). Similar to β_2 agonists, it is expected that this finding is a class effect and may be conserved with regular tiotropium use. However, one previous study with higher doses of ipratropium for a longer duration reported significant hyperresponsiveness to methacholine 24-hours after treatment withdrawal (Newcomb et al., 1985). Perhaps regular use of a muscarinic antagonist that stays bound to airway muscarinic receptors longer, such as tiotropium, may lead to bronchoprotective tolerance and enhanced allergen responsiveness.

An alternative explanation for the increased allergen responsiveness observed following tiotropium treatment could be enhanced mast cell degranulation as mast cells are the predominant source of bronchoconstricting mediators during the EAR. Reinheimer et al identified inhibitory M_1 muscarinic receptors on mast cells in isolated human bronchi (Reinheimer et al., 2000). Acetylcholine release from proximal non-neuronal cells onto mast cells inhibits histamine release via M_1 stimulation (Reinheimer et al., 1997). *In vitro* lung tissue investigations have shown both enhanced histamine release and no effects with muscarinic antagonist treatment (Kohno et al., 1989; Reinheimer et al., 1997). Despite being kinetically selective for M_3 receptors, tiotropium retains significant binding affinity for the M_1 receptor with a dissociation half-life of 10.5-hours (Kistemaker & Gosens, 2015). The EAR may have been slightly enhanced by tiotropium with the removal of cholinergic inhibition on mast cells. Enhanced mast cell degranulation is thought be involved in the significant worsening of

asthmatic responses to allergen with regular β_2-agonist use (Cockcroft et al., 1993; Cockcroft et al., 1995). The slight increase in allergen responsiveness with tiotropium could be due to a balancing of beneficial anti-inflammatory effects and detrimental effects on mast cells. It is theorized that antagonism of the M_3 receptor on dendritic cells would attenuate acetylcholine-enhanced polarization of dendritic cells to a type 2 promoting profile (Gori et al., 2019; Gori et al., 2017). The epithelial cytokine, TSLP, also demonstrates polarization of dendritic cells to a type 2 profile that promotes maturation of CD4+ T helper Type 2 lymphocytes. In contrast to tiotropium, anti-TSLP biologic therapy is clinically efficacious against allergen-induced asthmatic responses, eosinophil recruitment and reduces exhaled nitric oxide levels (Gauvreau, Hohlfeld, et al., 2020; Gauvreau et al., 2014). Tiotropium was theorized to elicit similar beneficial effects against allergen-induced responses. However, if acetylcholine modulates mast cell degranulation in addition to its proinflammatory role on dendritic cells, muscarinic antagonism of these dual functions could elicit neither a beneficial or detrimental effect, as seen (slight effect observed rather than strong inhibition or enhancement). This explanation relies heavily on data from animal model investigations on the non-neuronal cholinergic signalling system which does not always translate to in vitro human lung tissue findings, therefore this interpretation is cautioned (Kohno et al., 1989; Reinheimer et al., 2000). Unfortunately, the present study findings are limited to clinical data, and we can only infer mechanistic effects.

The trend toward increased allergen responsiveness following tiotropium treatment is further supported by analysis of the maximum fall in FEV_1 and FEV_1 recovery from allergen (i.e. progression to a late response). When the dose of allergen administered is kept constant, the maximum percent fall in FEV_1 is reproducible for the EAR and LAR (Inman et al., 1995). The primary endpoint of the present study was EAR $PD_{20,}$ and it was not required to keep the dose of allergen administered constant; a benefit of performing an EAR investigation (i.e. the ability to quantitate a dose response following pharmacological intervention). However, in the current investigation six participants responded slightly differently to the same dose of allergen after treatment. The mean maximal fall in FEV_1 in these individuals was 23.8% with tiotropium and 20.5% with placebo treatment providing a signal, albeit weak, to a worsened response to allergen following tiotropium pre-treatment. Tiotropium treatment also appears to cause a trend towards LAR development, largely influenced by two participants. These two participants experienced decreases in FEV_1 of 15.2% and 15.6% with tiotropium and only a 9.9% and 0.3% fall with

placebo, respectively. Based on these observations, the effect of tiotropium treatment on allergen-induced late asthmatic responses be further investigated.

The effect of tiotropium on allergen-induced inflammation remains inconclusive. Interpretation of these data is cautioned as only six participants received the same dose of allergen and allergen-induced inflammatory responses may be dose-dependent (Cockcroft & Davis, 2008). Five of eight sputum producers received different doses of allergen, all receiving less allergen during the tiotropium treatment period. Therefore, the slight differences in mean sputum eosinophils observed between the two treatments could be partially due to the dose of allergen. While late inflammatory sequelae are associated with the LAR, our pilot study aimed to observe a signal for anti-inflammatory efficacy of tiotropium at 5-hours after allergen. This study recruited all-comers with early responses, including dual responders. Therefore, the study population may not have much underlying allergic and eosinophilic inflammation, as demonstrated by a low/normal (< 25ppb) to intermediate (25-50ppb) mean baseline FeNO around 30ppb (Table 4.2) (Dweik et al., 2011). Although, sputum differential cell counts from a portion (8 participants) of the study population show relatively high eosinophilia (> 3% of total cell counts) (Figure. 4.3), at least two were suspected dual responders based on the present study and historical laboratory data. However, significant increases in sputum eosinophils after allergen were replicated in isolated early responders at 5-hours after allergen. This further supports the caution of extrapolated non-neuronal cholinergic animal model data as tiotropium has been shown to attenuate eosinophil infiltration after allergen in various ovalbumin challenge animal models (Bos et al., 2007; Bosnjak et al., 2014; Kang et al., 2012). John-Schuster et al found no change in eosinophil infiltration with tiotropium and in M_3-knockouts following house dust mite challenge in mice and explained the contradictory findings from use of a human-relevant allergen (John-Schuster et al., 2017). In our study, tiotropium did not inhibit allergen-induced eosinophil infiltration, suggesting that modulation of DC-polarization was not significant enough to reduce proinflammatory cytokines involved in the recruitment of eosinophils. This theory could be supported by immunohistochemistry analysis of sputum samples to quantify proinflammatory cytokine levels. Sputum samples from the present study will be stored for future investigation to confirm or refute this theory.

FeNO levels stayed constant throughout the study during both treatments, despite allergen inhalation. It is well documented (Gauvreau et al., 2014; Nomani et al., 2016; Rolla et al., 2011; Taylor et al., 1998) that FeNO is increased at 7- and 24-hours after allergen inhalation for dual and isolated late responders. However, Taylor et al found a trend towards increased FeNO as early as 2-hours after allergen in dual responders. Isolated early responders may not exhibit significant increases in FeNO until 21-hours after allergen (Kharitonov et al., 1995). The trend towards decreased FeNO at 5-hours after allergen in our study may be explained by reduced airway calibre following allergen as bronchoconstriction has been shown to decrease FeNO following bronchoprovocation challenges (Haccuria et al., 2014; Tadaki et al., 2009). The non-significant trend towards increased FeNO after 1-week of tiotropium treatment by 5.4ppb compared to an increase of 1.2ppb with placebo may suggest increases in underlying inflammation prior to the allergen-inhalation challenge. If this is the case, sputum differential cell counts at baseline and after regular tiotropium could provide more insight into inflammatory status with regular tiotropium use.

A study limitation that could have affected the outcome was unobserved administration of treatment doses #2-#7 by participants outside of the laboratory. If doses of tiotropium treatment were missed, the drug effect could be underestimated. However, at the final visit of each treatment period, all participants verbally stated no doses were missed. This was supported by a slight but non-significant mean increase in FEV_1 for the study population after 1-week of tiotropium treatment, prior to the final treatment dose (Table 4.4) that was significantly different from placebo treatment.

Table 4.4 *Mean FEV_1 (L) at baseline and after tiotropium versus placebo treatment.*

	Baseline	1-Week of Tx	30-minutes After Final Dose
Tiotropium	3.37 [0.34]	3.46 [0.42]	3.55* [0.48]
Placebo	3.47 [0.39]	3.36** [0.35]	3.42 [0.44]

Abbreviations: Tx: treatment; [SE]; *within tiotropium treatment significance: $p < 0.02$ compared to baseline and 1-week Tx timepoints; **within placebo treatment significance: $p < 0.01$ compared to baseline; Between treatment significance occurred between the 1-week Tx timepoint ($p = 0.03$) and 30-minutes after the final dose ($p = 0.02$) for tiotropium versus placebo treatment.

Bronchodilation from tiotropium in the well-controlled mild allergic asthmatic population was found in the present study, outside of study endpoints (Table 4.4). Significant bronchodilation was seen 30-minutes following the final dose of tiotropium by 180mL from baseline ($p = 0.02$) and 90mL ($p = 0.01$) from the pre-dose levels. This effect did not occur following placebo treatment. However, a slight but significant ($p < 0.01$) decline in mean FEV_1 of 110mL occurred following 1-week of placebo treatment. FEV_1 improvement in this population aligns with the beneficial lung function improvements seen in severe asthmatics when tiotropium is added on (*Spiriva® Respimat® Product Monograph*, 2017). If bronchodilation is conserved between these two populations, then the worsening trend with tiotropium and allergen-induced responses could occur in severe allergic asthmatics. Effects seen in clinical trials on mild allergic asthmatics are often extrapolated to the severe allergic asthma population. Tiotropium is currently indicated in severe asthma as an add on therapy in addition to ICS/LABA therapy in cases of poor control (*Spiriva® Respimat® Product Monograph*, 2017). Based on the present findings, we suggest further investigation into the regular use effect of tiotropium and the effect of tiotropium on allergen-induced late asthmatic responses and late sequelae to further elucidate the safety and efficacy of LAMA in allergic asthma.

5.0 GENERAL DISCUSSION

This work has demonstrated the regular use effect of inhaled muscarinic antagonists on methacholine responsiveness (SAMA investigation) and allergen responsiveness (LAMA investigation). The results of these clinical trials contradict each other regarding safety of regular use of muscarinic antagonists. Bronchoprotection provided by single dose (40μg) ipratropium against methacholine-induced bronchoconstriction was maintained following 1-week of regular ipratropium (40μg thrice daily). This suggests that tolerance does not occur following regular stimulation of airway muscarinic receptors. In contrast, tolerance rapidly occurs to the bronchoprotective effect of β_2-agonists after regular use (Bhagat et al., 1995; Bhagat et al., 1996; Boulet et al., 1998; Cheung et al., 1992; Drotar et al., 1998; O'Connor et al., 1992; Stewart et al., 2012). Although originally observed with regular use of short-acting β_2-agonists, tolerance also develops with regular use of long-acting β_2-agonists and therefore, the development of tolerance appears to be a class effect.

While it is unknown whether bronchoprotection against methacholine-induced constriction will be maintained following regular LAMA use, we previously (Cropper et al., 2020) assumed that this would be a class effect and that tolerance would not occur. The subsequent discovery of regular use tiotropium (5μg once daily for 1-week) and worsened early asthmatic responses to allergen suggests this may not be the case. Regular β_2-agonists significantly enhance airway hyperresponsiveness to allergen during the EAR and LAR, an effect that coincides with the development of tolerance to bronchoprotection (Cockcroft et al., 2007; Cockcroft et al., 1993; Cockcroft et al., 1995). Therefore, our assumption that the absence of tolerance may occur with regular LAMA use could be wrong based on the observed allergen hyperresponsiveness. A previous study on regular SAMA use also reported no development of tolerance to the bronchoprotective effects of ipratropium after 2-weeks of regular use (60μg four times daily for 3-weeks) (Newcomb et al., 1985). However, they found methacholine hyperresponsiveness 24-hours after treatment withdrawal, suggesting that muscarinic receptors may have been upregulated or sensitized. Our study used a lower dose of ipratropium (40μg versus 60μg) for a shorter length (1-week versus 3-weeks) and therefore, the lower dose may have contributed to the lack of tolerance and hyperresponsiveness observed. It is of great interest whether regular use of tiotropium, with an effective half-life of 34-hours, will maintain bronchoprotection against

methacholine or whether tolerance will develop. We suggest future investigation into this topic, as it will help inform guidelines on usage of muscarinic antagonists as well as provide insight into the increased allergen responsiveness we observed with regular tiotropium.

A potential limitation of this work is the neutralizing effect of mean data that can minimize individual responses. Individual responses may become of greater importance in clinical research with the shift to personalized medicine. Previously reported variability in SAMA-treated individuals' response to allergen, may be due to underlying factors that predispose some to better responses with cholinergic modulation (Cockcroft et al., 1978; Orehek et al., 1975; Yu et al., 1972). The present clinical trials used a relatively small populations, making the identification of sub-groups with alternative responses difficult.

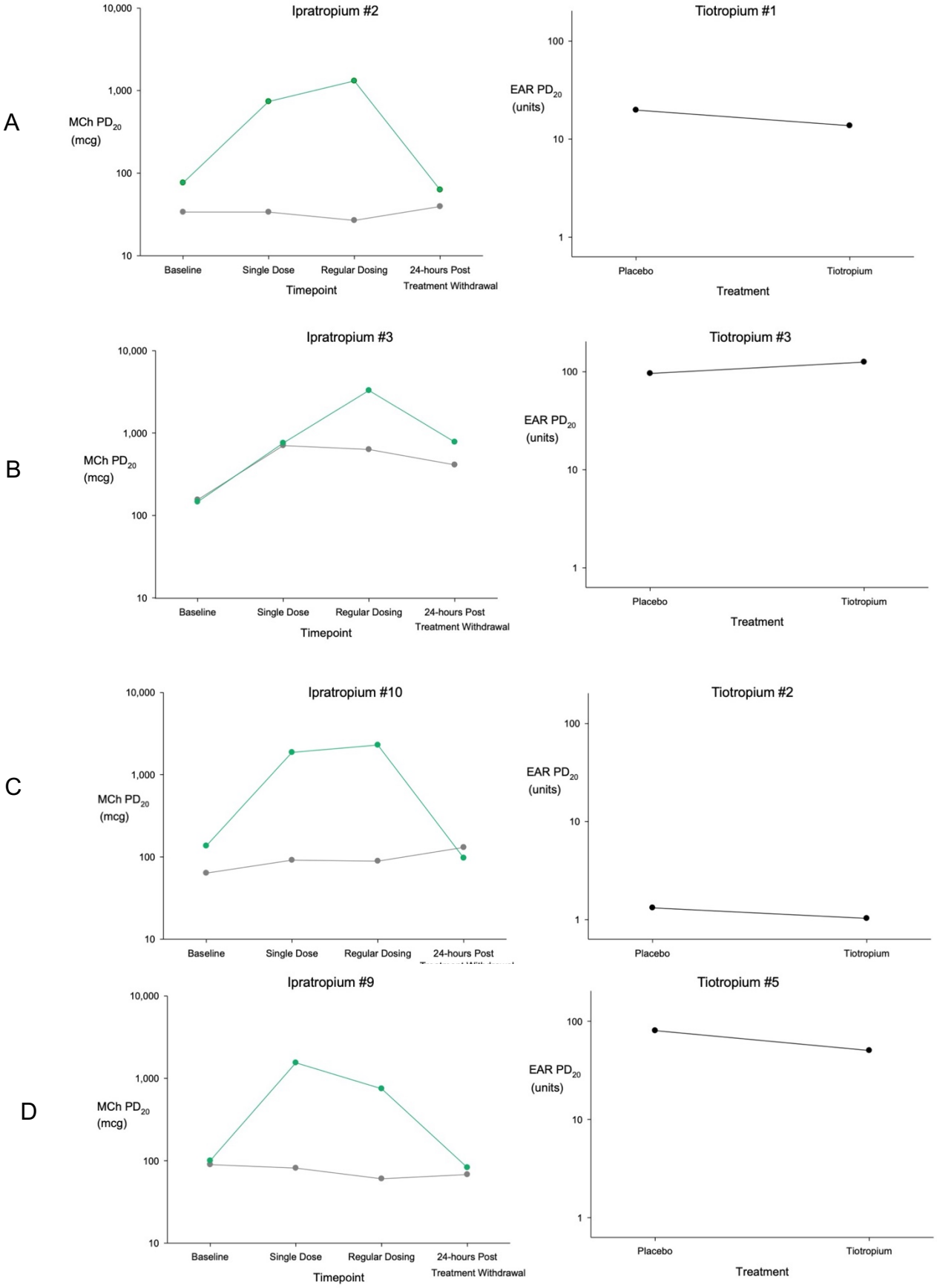

Figure 5.1 *Individual allergen and methacholine PD_{20} data for participants involved in both studies. Ipratropium (green), placebo (grey or black) and tiotropium (black) treatments shown.*

Four participants were involved in both present clinical trials and therefore their individual results under the two conditions are of interest (Fig. 5.1). Participants in panels A and C serve as representative examples for the trends observed in the mean data for both studies. Under regular use of ipratropium, these two participants did not develop tolerance to bronchoprotection with ipratropium or hyperresponsiveness to methacholine. With regular use of tiotropium, these two participants demonstrated slightly increased allergen responsiveness. Panel C represents a participant who had unique results in both studies. Following regular use of ipratropium, this participant exhibited stronger bronchoprotection against methacholine, evidenced by a greater MCh PD_{20} after 1-week of ipratropium (MCh PD_{20} = 753.6μg) in comparison to a single dose of ipratropium (MCh PD_{20} = 146.2μg). This participant demonstrated a strong placebo response, evidenced by a clinically significant dose shift (DS) in MCh PD_{20} after a single dose of ipratropium (DS = 2.4 doubling doses) and placebo treatments (DS = 2.2 doubling doses). Following regular tiotropium use, this participant required a greater dose of allergen (EAR PD_{20} = 125.7 allergen units) to induce the same fall in FEV_1 as with placebo treatment (EAR PD_{20} = 96.2 allergen units). This was one of only three participants with reduced airway hyperresponsiveness to allergen after tiotropium treatment. Given the strong bronchoprotection placebo response seen under the ipratropium study, it is difficult to ascertain whether tiotropium had an isolated beneficial effect in this participant or if other factors are influencing this response. The mean data can be skewed by individual responses such as those displayed by the participant in panel B. Panel D represents a participant with a unique response to ipratropium and the reported response (increased allergen responsiveness) with tiotropium. After 1-week of ipratropium, this participant experienced less bronchoprotection (DS = 2.9) compared to after a single dose (DS = 4.0) and therefore tolerance may have occurred within this individual. Slight hyperresponsiveness to methacholine also occurred 24-hours after ipratropium withdrawal. Following 1-week of tiotropium treatment, this individual experienced increased allergen responsiveness, similar to the mean data. These participants demonstrate the potential for unique individual responses that may be due to important pathophysiological mechanisms, unexplained by mean data. With the shift towards personalized medicine, it may become important to incorporate case study and large sample size asthmatic clinical trials to identify sub-groups with differential responses.

Phenotyping and endotyping of asthma may improve treatment choice by identifying pathophysiological mechanisms underlying the disease. For example, sputum differential cell analyses identifying underlying eosinophilic airway inflammation in severe asthmatics indicates the use of an anti-IL-5 monoclonal antibody may be effective. Accumulating data, including the present data, suggest differing significance of cholinergic signalling among mild asthmatics. We suggest further investigation into the various factors contributing to differential responses to asthma therapies, including muscarinic antagonists. Furthermore, identification of specific features and biomarkers associated with asthma endotypes could improve diagnosis and management of asthma.

This work provides a basis for further investigation into asthma management with inhaled muscarinic antagonists and their regular use effects. We suggest future clinical evaluation of the regular use effect of inhaled tiotropium on methacholine responsiveness and a single dose of tiotropium on allergen responsiveness to expand on the present findings. A major limitation of this work is the inability to determine mechanisms underlying the observed clinical effects. Further human tissue investigations with regular muscarinic antagonists may provide insight into underlying cellular events contributing to our findings.

In summary, the muscarinic antagonists, ipratropium bromide (short-acting) and tiotropium bromide monohydrate (long-acting) were found to have opposed regular use effects on the bronchoconstricting stimuli, methacholine, and allergen, respectively. It is unclear whether this observed difference is due to the duration of action (short versus long-acting) or the body's response to the stimulus (methacholine versus allergen). Non-neuronal cholinergic signalling remains an interesting and somewhat enigmatic player in immune responses that should continue to be studied in the human model. Future investigations may help guide clinical use of muscarinic antagonists as asthma management therapies.

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7.0 APPENDICES

Appendix A: CJRCCSM Publication of Ipratropium Study Manuscript

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ORIGINAL RESEARCH

Regular use effect of inhaled ipratropium bromide and methacholine responsiveness in well-controlled asthma

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ABSTRACT

RATIONALE: Short-acting β_2-agonists provide significant bronchoprotection to inhaled methacholine in individuals with asthma. Regular use of β_2-agonists results in the development of tolerance to the bronchoprotective effect. A relatively minimal amount is known regarding the development of tolerance following regular use of short-acting muscarinic antagonists.

OBJECTIVES: The current study was undertaken to assess bronchoprotection after regular use of inhaled ipratropium bromide ("ipratropium") and after treatment withdrawal.

METHODS: Twelve adult participants with mild asthma were assigned to treatment in this double-blind randomized placebo-controlled crossover study. Ipratropium, 40 µg thrice daily, and matching placebo were administered for 1 week (22 doses). Methacholine challenges were performed at baseline, 30-min post first and last dose and 24-h after the final dose. A minimum 7-day washout period separated the treatments.

MEASUREMENTS AND MAIN RESULTS: Significant bronchoprotection, measured as an increase in methacholine PD_{20} of 4.3 doubling doses (95% CI 3.62–5.06), occurred following the first dose of ipratropium. Bronchoprotection increased slightly to 4.5 doubling doses (95% CI 3.80–5.27) following regular use. Dose shifts following placebo were significantly lower at 0.23 (95% CI −0.30–0.75) and 0.11 (95%CI −0.63–0.85) after the first and last dose respectively ($p < 0.001$ vs. ipratropium for both). Changes from baseline methacholine PD_{20} 24-h after treatment withdrawal were not significant between treatments ($p = 0.19$).

CONCLUSIONS: Our data provide evidence that regular use of inhaled ipratropium does not result in loss of bronchoprotection or lead to rebound hyperresponsiveness following treatment withdrawal. Muscarinic antagonists appear to have a superior safety profile over β_2-agonist use in the treatment of asthma.

RÉSUMÉ

JUSTIFICATION: Les β2-agonistes à courte durée d'action procurent une bronchoprotection significative à la méthacholine inhalée chez les personnes asthmatiques. L'utilisation régulière de β2-agonistes entraîne le développement d'une tolérance à l'effet bronchoprotecteur. On en sait relativement peu sur le développement de la tolérance suite à l'utilisation régulière d'antagonistes muscariniques à courte durée d'action.

OBJECTIFS: Cette étude a été entreprise pour évaluer la bronchoprotection après une utilisation régulière de bromure d'ipratropium inhalé (« ipratropium ») et après l'arrêt du traitement.

MÉTHODES: Douze participants adultes souffrant d'asthme léger ont été assignés au traitement dans cette étude transversale randomisée en double aveugle contre placebo. De l'ipratropium, à raison de 40 µg trois fois par jour, et un placebo correspondant ont été administrés pendant une semaine (22 doses). Des provocations à la méthacholine ont été réalisées au départ, 30 minutes après la première et la dernière dose et 24 heures après la dernière dose. Une période de sevrage minimale de sept jours a séparé les traitements.

MESURES ET RÉSULTATS PRINCIPAUX: Une bronchoprotection significative, mesurée comme une augmentation de la méthacholine PD_{20} de 4,3 doses doublées (IC à 95% 3,62-5,06), est survenue après la première dose d'ipratropium. La bronchoprotection a légèrement augmenté à 4,5 doses doublées (IC à 95% 3,80 - 5,27) après une utilisation régulière. Les changements de dose après le placebo étaient significativement plus faibles à 0,23 (IC à 95% −0,30-0,75) et 0,11 (IC à 95% −0,63 - 0,85) après la première et la dernière dose respectivement ($p < 0,001$ par rapport à l'ipratropium pour les deux). Les changements par rapport à la méthacholine PD_{20} de base 24 heures après l'arrêt du traitement n'étaient pas significatifs entre les traitements ($p = 0,19$).

CONCLUSIONS: Nos données fournissent des preuves que l'utilisation régulière d'ipratropium inhalé n'entraîne pas de perte de bronchoprotection ou de rebond d'hyperréactivité après l'arrêt du traitement. Les antagonistes muscariniques semblent avoir un profil d'innocuité supérieur à l'utilisation des β2-agonistes dans le traitement de l'asthme.

KEYWORDS

Asthma; ipratropium; methacholine; bronchoprotection; tolerance

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Introduction

Asthma is a heterogeneous respiratory disease characterized in part by airway inflammation and airway hyperresponsiveness to direct acting stimuli (e.g., methacholine). The bronchoconstriction associated with asthma can be treated with various bronchodilators that act on either adrenergic or muscarinic receptors. Most currently used adrenergic bronchodilators selectively bind β_2 receptors of the airways to relax airway smooth muscle and induce bronchodilation. Muscarinic antagonists bind airway smooth muscle muscarinic subtype 3 (M_3) receptors as the predominant mechanism for inhibition of bronchoconstriction. M_3 receptors elicit airway smooth muscle contraction when stimulated by endogenous acetylcholine (ACh) or inhaled methacholine (MCh). MCh is a structural analog of ACh commonly used to induce bronchoconstriction in research and clinical settings. Muscarinic antagonists and β_2-agonists elicit a bronchoprotective effect against MCh-induced constriction via competitive or functional antagonism, respectively.

Tolerance to the bronchoprotective effect following regular use of inhaled β_2-agonists, including both the short-acting agonist salbutamol and the long-acting agonist salmeterol, has been well documented,[1–4] and occurs quite rapidly.[5–7] A decrease in the number of plasma membrane β_2 receptors or "receptor downregulation" has been shown to play a role.[8] Tolerance to the vasoactive properties of various alpha and beta adrenergic receptor antagonists via receptor upregulation, or an increase in receptor number following prolonged use, is common in the treatment of cardiovascular disorders.[9–11] A minimal amount is known, however, regarding the development of tolerance to inhaled muscarinic antagonists but receptor upregulation has been observed in other tissues, under different conditions.[12–14] The data showing changes in receptor densities following regular use of other drugs targeting G protein coupled receptors together with evidence that supports muscarinic receptor upregulation suggest the possibility of receptor upregulation and subsequent loss of bronchoprotection following regular use of inhaled ipratropium. Despite the supportive data, a previous study in individuals with asthma failed to elicit a tolerance response to the bronchoprotective effect following regular high dose use of inhaled ipratropium but did document a transient rebound hyperresponsiveness to methacholine 24-h after treatment withdrawal.[15] The outcomes of the previous study may have been influenced by study design. We therefore undertook the current investigation using standardized methodology and clinically indicated doses to assess the effect of regular use of inhaled ipratropium on both bronchoprotection and rebound hyperresponsiveness to methacholine in individuals with well-controlled asthma.

Materials and methods

Participants

Twelve nonsmoking participants at least 18 years of age with well-controlled mild asthma were recruited. Participants were required to have stable lung function evaluated based on forced expiratory volume in one second (FEV_1) $\geq$ 65% of predicted and baseline dose of methacholine causing a 20% fall in FEV_1 (methacholine PD_{20}) $\leq$ 200 µg (equivalent to a Wright 2 min tidal breathing PC_{20} of 8 mg/mL).[16] Participants were allowed to use salbutamol as a rescue bronchodilator, provided it was not used within 6-hours of a study visit. Daily inhaled corticosteroid (ICS) use was also allowed if dosing was consistent and stable for at least 4 weeks (n = 1). Individuals were ineligible if they had used a long-acting β_2-agonist or a muscarinic antagonist in the 30 days prior to the study. All other asthma controller or bronchodilator treatments were not allowed. Individuals also had to be devoid of any upper or lower respiratory tract infection for at least 4 weeks prior to study entry. In addition, individuals were ineligible if they had been exposed to an allergen in the 4 weeks prior to the study, were pregnant or lactating, had a diagnoses of diabetes or glaucoma or if they had cardiovascular, urinary, prostate or kidney problems. Written informed consent was obtained prior to performing any study related procedures. The study was approved by the University of Saskatchewan Biomedical Research Ethics Board and registered with clinicaltrials.gov as NCT#04167280.

Methacholine Challenge Test (MCT)

During each study visit, spirometry was used to establish a baseline FEV_1 using an nSpire KoKo® spirometer (Carestream Medical Ltd, Oakville, Ontario, Canada). Baseline lung function was evaluated based on FEV_1 and forced vital capacity (FVC) data using standardized equations.[17] Participants inhaled 0.5 mL of aerosolized saline by tidal breathing via an Aerogen Solo® vibrating mesh nebulizer (Canadian Hospital Specialties Ltd, Oakville, Ontario, Canada) until the entire volume was aerosolized. A shortened-spirometry maneuver was performed at 30-sec and 90-sec post-inhalation to capture FEV_1 only. Provocholine® (Methapharm, Inc., Brantford, Ontario, Canada) reconstituted with normal saline was used for methacholine inhalation. The MCT proceeded per the volumetric method[18] with doubling doses administered at 5-min intervals until a minimum fall in FEV_1 of 17% was achieved (at baseline challenge only) or until the maximum dose of methacholine had been administered (200 µg at baseline or 6400 µg for post-treatment tests). Methacholine PD_{20} values were interpolated[19] or extrapolated[20] using standard formulae.

Study design

The study followed a double-blind, randomized, placebo-controlled, crossover design. In randomized order, participants underwent a treatment period with the study drug, ipratropium, and a treatment period with a matching placebo. Treatment periods were separated by a minimum 7-day washout. During each treatment period, participants were required to take 2 puffs from the inhaler 3 times daily for 7 days. The first dose and a final dose (dose #22) on Day 8 were administered in the laboratory. Four MCTs were performed during each treatment period; 2 on Day 1 (single dose effect), one on Day 8 (regular use effect) and one on Day 9 (treatment withdrawal effect). The first MCT was

Table 1. Participant baseline demographics.

P#	Sex	Age	Height (cm)	Weight(kg)	Placebo FEV_1 (L) (% predicted)	IB FEV_1 (L) (% predicted)	Placebo Baseline PD_{20}	IB Baseline PD_{20}
1	M	21	178	68	3.41 (78)	3.60 (83)	154.2	228.6
2	M	43	178	89	3.49 (84)	3.60 (87)	33.8	76.3
3	M	19	178	91	3.92 (91)	3.51 (81)	155.0	146.2
4	F	18	155	64	2.65 (99)	2.65 (100)	149.5	102.6
5	M	73	168	66	2.02 (75)	2.03 (75)	28.9	35.9
6	M	35	196	85	4.95 (92)	4.93 (92)	56.5	38.4
7	F	23	162	66	3.45 (105)	3.66 (111)	10.0	15.3
8	M	18	177	59	4.21 (92)	4.20 (92)	196.4	74.9
9	M	22	167	68	3.59 (97)	3.57 (96)	89.7	100.0
10	M	29	173	80	3.86 (91)	4.10 (97)	63.6	136.2
11	M	28	175	71	3.42 (78)	3.38 (77)	155.1	183.9
12	F	19	160	45	2.53 (79)	2.61 (81)	69.0	123.0
Mean:	75%	29	172.3	71	3.46 (88)	3.49 (89)	72.4[a]	85.1[a]
	M	[16.3]	[10.5]	[10.8]	[0.78]	[0.78]	42–129[b]	51–138[b]

P#, participant number; FEV_1, forced expiratory volume in 1 second; IB, ipratropium; PD_{20}, dose of methacholine causing a 20% fall in FEV_1; [SD]: [a]Geometric mean; [b]95% confidence interval.

used to determine eligibility and establish a baseline for the remainder of the treatment period. A 30-min recovery period followed the first MCT prior to administration of the first dose. The second and third MCT's commenced 30-minutes after dosing. The final MCT was completed 24-h after the final dose. All MCT were completed at the same time of day ± one hour.

Study drug and blinding

Kits comprised of one ipratropium inhaler (Atrovent®) and one placebo inhaler, both pressurized metered dose inhalers, were prepared by one of the investigators not involved in data collection. One of the 2 canisters was labeled as treatment 1 and the other as treatment 2. A randomization code was sealed in an envelope. Participant 1 received kit 1, treatment 1 first and treatment 2 second. Subsequent participants received subsequent kits in numerical order, each receiving treatment 1 first and treatment 2 second.

Data analysis

Methacholine PD_{20} data were log transformed prior to analyses. Treatment effect was determined by calculating the dose shift in methacholine PD_{20} and reported as doubling doses. The following formula was used: dose shift = $[(\log PD_{20post\text{-}dose} - \log PD_{20baseline})/0.3]$.[1] Between treatment dose shifts and within treatment PD_{20} differences were compared using Student's paired t-test (alpha 0.05) and Statistix 9 software (Analytical software, Tallahassee, Florida). A sample size of 12 participants provided a study power of >90% to detect a one dose difference in methacholine PD_{20}.

Results

Participants

A total of 22 participants were enrolled in the study, 10 of which failed to meet methacholine PD_{20} inclusion criteria (i.e. had baseline methacholine PD_{20} > 200 μg). One participant with a baseline PD_{20} of 228.6 μg was included in the investigation based on historical data (i.e., methacholine PD_{20} recently <200μg) and on level of commitment to participating in a clinical trial (i.e., known to be reliable). Eleven participants followed the study to completion and one additional participant, the only participant using regular ICS (budesonide 200 μg bid), was unable to finish treatment 2 (placebo) due to safety measures taken during the COVID-19 pandemic (Table 1). No unexpected or serious adverse effects occurred. Mean baseline lung function and geometric mean methacholine PD_{20} data were similar for both treatments (Table 1).

Bronchoprotection, tolerance and rebound hyperresponsiveness

On average, single dose ipratropium (40 μg) significantly decreased airway responsiveness to inhaled methacholine by 4.3 doubling doses (i.e. increased methacholine PD_{20} by more than 16-fold) versus a doubling dose shift of 0.23 following placebo ($p < 0.001$) (Figure 1). After 7 days of regular use the magnitude of bronchoprotection increased slightly to 4.5 doubling doses following ipratropium and decreased slightly to 0.11 doubling doses following placebo ($p < 0.001$) (Figure 1). No rebound hyperresponsiveness was detected as dose shifts from baseline to 24-h were −0.15 and 0.34 doubling doses following ipratropium and placebo withdrawal, respectively ($p = 0.19$) (Figure 1). Individual methacholine PD_{20} at all time points for both treatments are shown in Figure 2. Geometric mean PD_{20} data are tabulated in Table 2.

Discussion

In our study of twelve mild well controlled asthmatics, regular use of ipratropium did not result in tolerance to bronchoprotection or rebound hyperresponsiveness following treatment withdrawal. The literature in this area is sparse. We are aware of only one publication from 35 years ago in which tolerance to methacholine induced bronchoconstriction and rebound hyperresponsiveness following regular use of ipratropium were studied.[15] In this previous investigation,

tolerance did not develop following regular use of high dose ipratropium (60 μg 4 times daily for 3 weeks), however, transient rebound hyperresponsiveness was documented 24-h after treatment withdrawal. A major limitation of the previous study was that the highest concentration of MCh administered was only 25 mg/mL which was not high enough to elicit a 20% fall in FEV_1 and generate a dose response following treatment. Failure to quantify maximal bronchoprotection to MCh may have skewed the results and underestimated the loss of bronchoprotection following regular use. Additional limitations of the previous study, include the absence of a placebo arm (possible bias due to not accounting for a placebo effect), the small sample size (potentially underpowered with only 9 participants) and the use of dosimeter deep inhalation methodology (direct comparison with our current data may not be applicable due to different testing methodologies). Nonetheless, the findings of the previous study (3 puffs 4 times a day for 3 weeks; high dose) and our current study (2 puffs 3 times a day for 1 week; low dose) suggest regular use of ipratropium does not result in loss of bronchoprotection to methacholine. These data, taken together with our knowledge of dose response and the development of tolerance following regular use of β_2 agonists (dose independent[2] and rapid onset[5–7]) suggest that increasing the dose or the duration of dosing with ipratropium are unlikely to result in the development of tolerance. This should be confirmed by prospective investigation.

The absence of tolerance following regular use ipratropium is in stark contrast to the loss of bronchoprotection observed following regular use of both short and long acting β_2-agonists. An early investigation by O'Connor et al. showed a small but significant reduction in bronchoprotection against methacholine following 7 days of high dose terbutaline.[1] Bhagat et al. showed close to one doubling dose increase in methacholine responsiveness across all doses of salbutamol (200, 400 and 800 µg) after one week of daily treatment.[2] Tolerance has also been shown following 8 weeks[3] and 4 weeks[4] of twice daily salmeterol. Subsequent investigations documented the reduced efficacy to β_2 agonist bronchoprotection to occur quite rapidly. In the case of salmeterol, statistically significant decreases of 44% and 37% in geometric mean methacholine PC_{20} occurred after just 2 50-μg doses;[5,6] with twice daily salbutamol (200 µg q12h) loss of bronchoprotection was observed after just 7 doses (i.e., 3.5 days).[7] As a side note, the addition of inhaled corticosteroids as a preventative measure against the loss of bronchoprotection proved ineffective following salmeterol use[4,21] but beneficial, *ex vivo*, following salbutamol use.[22] Recent clinical trial data obtained from a larger sample challenges the previous negative findings not only with respect to the beneficial effects of

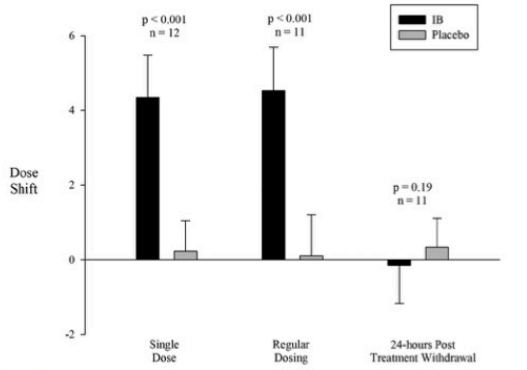

Figure 1. Dose shift (doubling dose) comparisons between ipratropium (IB) and placebo treatments after a single dose (40 µg), regular dosing (40 µg thrice daily for 7 days) and 24-hours after treatment withdrawal. IB data is a full data set (n = 12); Placebo data set is n = 12 for single dose and n = 11 for regular dosing and 24-hour post treatment withdrawal. Sample size noted on graph relates to statistical analyses.

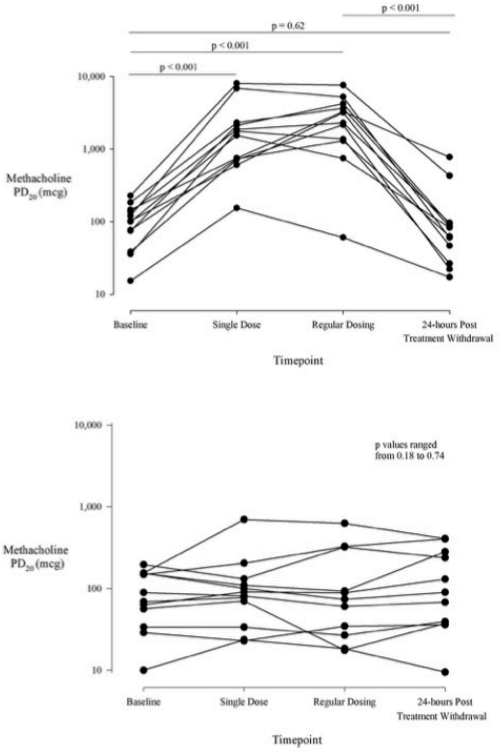

Figure 2. Individual log methacholine PD_{20} data after single dose (2 puffs), regular dosing (2 puffs thrice daily) and following treatment withdrawal (24-hours) for ipratropium (n = 12, top) and placebo (n = 11, bottom) treatments.

Table 2. Geometric mean PD_{20} (μg) data.[a]

	Baseline	Single Dose	Regular Dosing	24-hour Post Tx
Ipratropium	85 (51–138)	1698 (813–3467)	1905 (871–4266)	76 (37–155)
Placebo	72 (42–129)	85 (47–155)	79 (35–182)	93(42–209)

(95% confidence interval) [a]divide PD_{20} by 25 to estimate equivalent Wright 2 minute tidal breathing PC_{20} value.

concomitant ICS but also to the prevalence of tolerance and the role of β_2 receptor downregulation as a potential mechanism.[23] One participant in our study was using ICS and this individual produced data consistent with that of the study cohort (i.e., significant single dose bronchoprotection that was sustained after regular use and a slight increase in response 24 hours after treatment withdrawal) suggesting that concomitant ICS use would be unlikely, and not expected, to alter our observed study outcome.

Our findings are inconsistent with the Newcomb study data in that we did not observe rebound hyperresponsiveness to methacholine following treatment withdrawal. A possible explanation for the contradictory findings between our current study and the previous study may be attributed to differences in dosing as a higher dose for a longer duration (60 µg given 4 times daily for 3 weeks compared to our study design of 40 µg given 3 times daily for 1 week) may have led to a state of supersensitivity following treatment withdrawal.

The absence of the development of tolerance in our study suggests muscarinic receptor upregulation did not occur following one week administration of clinically relevant dosing of ipratropium. Given that receptor upregulation is a well-documented phenomenon following prolonged antagonism such as occurs after chronic use of adrenergic receptor antagonists[9–11] and with evidence that muscarinic receptor upregulation occurs in non-lung tissue[12–14] and given that receptor downregulation occurs both quickly and at clinically relevant doses with β_2 agonists within the lung, it is unclear why loss of bronchoprotection to methacholine was not observed in our investigation. One possible explanation could be poor dosing compliance as ipratropium was self-administered for doses 2 through 21 however we verbally confirmed dosing compliance with participants following regular dosing (i.e. in the laboratory prior to administration of the final dose) and no participant reported any missed doses. Another possible and important consideration for a negative study is that of sample size or study power. We can rule this out as well as a sample size of 12 individuals provides a study power of 90% to detect a one dose difference in methacholine PD_{20}.[24]

Our findings are limited to clinical data and any mechanistic effects can only be inferred. Evidence has shown that receptor antagonism with some beta blockers does not lead to receptor upregulation and can actually lead to a decrease in receptor density.[25] Another relatively new phenomenon is that of biased antagonism, where the receptor is blocked in a competitive manner but intracellular signaling pathways responsible for regulating receptor numbers (i.e., β arrestin activity) are not affected.[26] Given the promising data that biased antagonists have produced, one wonders if a similar strategy (i.e., biased agonism) toward β_2 receptors in the lung would prove beneficial in preventing tolerance but maintaining the excellent bronchoprotective and bronchodilatory properties. While these mechanistic data are intriguing, the relevance to explaining the possible mechanistic rationale for the absence of tolerance following ipratropium use is limited (i.e., intrinsic sympathomimetic activity and small peptide molecules). *Ex vivo* muscarinic M_3 receptor binding assays using human lung tissue would be one method for determining whether or not receptor upregulation occurs on airway smooth muscle following prolonged ipratropium use. Consideration might also be given to the nonselective receptor binding properties of ipratropium and the potential role concomitant antagonism of muscarinic subtypes 1 and 2 may play in regulating M_3 receptor numbers. Given the class effect on the development of tolerance following regular use of β_2 agonists (i.e., loss of bronchoprotection occurs with both short and long acting beta agonists) a clinical investigation on loss of bronchoprotection following regular use of tiotropium may provide additional insight. Although anticipated to also be a negative study based on β_2 agonist data, prolonged receptor occupancy and receptor specificity for the M_3 subtype are rationale supporting a different outcome.

With respect to asthma, ipratropium is only indicated for acute use in the management of exacerbations, therefore, potential clinical implications from our findings relate to off label use in the routine management of asthma and, indirectly, to use in COPD where treatment with anticholinergics is both recommended and commonly used. In light of the well-documented detrimental effects of regular β_2-agonist use in the treatment of asthma, it may be useful to further investigate, or perhaps renew our interest in the mechanisms and clinical effects of targeting the cholinergic system and the use of muscarinic antagonists as alternative treatments.

Acknowledgments

The authors would like to thank all participants for their contribution.

Declaration of interest

The authors report no conflict of interest.

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Appendix B: BIO #1522 Certificate of Approval

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 31/Oct/2019

Certificate of Approval

Application ID:	1522
Principal Investigator:	Donald Cockcroft Department: Department of Medicine
Locations Where Research Activities are Conducted:	Asthma Research Lab Room 346 Ellis Hall, Canada
Student(s):	Kayla Cropper
Funder(s):	
Sponsor:	Asthma Research Lab
Title:	Regular Use Effect of Inhaled Ipratropium Bromide on Airway Responsiveness to Methacholine in Asthma
Protocol Number:	
Approved On:	29/Oct/2019
Expiry Date:	28/Oct/2020
Approval Of:	* Biomedical Application, Prospective, rec'd 24-Oct-2019 * Participant Information and Consent Form, Version October 21, 2019 (Revision 1) * Recruitment Advertisement, rec'd 24-Oct-2019 * PAWS Advertisement, rec'd 18-Sep-2019 * Research Protocol, rec'd 18-Sep-2019
Acknowledgment Of:	* "Methacholine Challenge Testing: Comparative Pharmacology" Journal Article
Review Type:	Full Board
Meeting Date:	02/Oct/2019
IRB Registration Number:	Not Applicable

1 / 2

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal risk projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Appendix C: BIO #1522 Certificate of Approval Amendment

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 09-Dec-2019

Certificate of Approval Amendment

Application ID: 1522

Principal Investigator: Donald Cockcroft Department: Department of Medicine

Locations Where Research Activities are Conducted: Asthma Research Lab Room 346 Ellis Hall, Canada

Student(s): Kayla Cropper

Funder(s):

Sponsor: Asthma Research Lab

Title: Regular Use Effect of Inhaled Ipratropium Bromide on Airway Responsiveness to Methacholine in Asthma

Protocol Number:

Approved On: 08/12/2019

Expiry Date: 28/10/2020

Approval Of:

* Protocol (tracked changes) Amend 1 18-Nov-2019
* Main Consent Form: Track Change Version Amend 1 18-Nov-2019
* Pharmacy ad Amend 1

Acknowledgment Of:

Review Type: Delegated Review

IRB Registration Number: Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Appendix D: BIO #1522 Participant Consent Form

UNIVERSITY OF SASKATCHEWAN

Participant Information and Consent Form

Title of Study: Regular use effect of inhaled ipratropium bromide on airway responsiveness to methacholine in well-controlled asthma

Principal Investigator:

Dr. Donald Cockcroft, MD FRCP
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Co-Principal Investigator:

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Student Researcher:

Kayla Cropper
Physiology and Pharmacology Honors Student
University of Saskatchewan
kje889@usask.ca

24-Hour Emergency Contact:

In the event of a last-minute change to your schedule, please call Kayla at or email at kje889@usask.ca.

Introduction: You are invited to participate in this research study. Your age (i.e. at least 18 years of age) and health status (i.e. mild asthma) make you a suitable representative of the population of interest.

Your participation is voluntary. It is entirely up to you to decide whether or not you wish to take part. If you wish to participate, you will be asked to sign this form. You may withdraw from the study at any time and you do not have to give any reasons for doing so.

If you do not wish to participate, it will not impact your current or future healthcare, academic standing, or employment.

Please take time to read the following information carefully. You may direct any questions or concerns to the study staff prior to and at any time during the study. Please feel free to discuss this with your family, friends or family physician before you decide.

UNIVERSITY OF SASKATCHEWAN

Agencies Contributing Funds and Resources to the Study: This study is being conducted by Dr. Don Cockcroft, Dr. Beth Davis and Kayla Cropper the student researcher. The Asthma Research Lab will be managing the cost of the study without financial aid from a third-party organization. The researchers and the University of Saskatchewan are not being paid to conduct this study.

Purpose of Study: This study is being conducted to determine if ipratropium bromide (e.g. Atrovent®) taken three times daily for seven days (i.e. 2 puffs in the morning, 2 puffs in the afternoon and 2 puffs in the evening) followed by a final dose on the morning of day 8 reduces the effectiveness of this medication. (i.e. does regular use of the medication lead to a decrease in the medication effect)

Background Information: Atrovent® belongs to a class of medications called bronchodilators. Bronchodilators, which include Atrovent® and salbutamol (Ventolin®), are often referred to as rescue medication and are used to quickly relieve wheezing and shortness of breath in asthma. Bronchodilators will also protect the airway from constricting when substances like methacholine are inhaled. Methacholine is a substance structurally similar to acetylcholine. Acetylcholine is a chemical in our body that causes bronchoconstriction (i.e. airway constriction making it hard to breath). Methacholine testing is used by doctors to help determine if an individual has a hyper-responsive airway. If an individual responds to inhaled methacholine, there is a chance the individual has asthma. Previous research has demonstrated that bronchodilators like salbutamol and salmeterol that relax airway smooth muscle and relieve bronchoconstriction, become less effective with frequent use. Very little is known regarding the effectiveness of Atrovent® following regular use.

Eligible Participants (12-15 required): To participate in this study, you must be at least 18 years old. Additional criteria for entering the study includes acceptable baseline lung function and a positive response to methacholine. The specifics regarding these criteria will be discussed with you. For now,. FEV_1 is the amount of air you can forcefully exhale during the first second of exhalation. This value must be at least 65% of what is predicted for your age, gender and height. PD_{20} is the dose of methacholine causing a 20% reduction in your FEV_1 and it must be 200ug or less.

You may not participate if you are pregnant, nursing, or suffer from heart problems. Please disclose if you are on any medications or have any pre-existing health conditions. Certain medications and health conditions may affect results of this study or may put your health at risk if you choose to participate. The study will require that you have not used Atrovent®, or similar acting medication, in the past 30 days. You will be allowed to use a salbutamol inhaler as needed; however, it cannot be used within 6 hours of lab visits. In an emergency situation, use the inhaler and contact Kayla to re-schedule your visit. Any current or recently taken medications will be reviewed and discussed. You are not permitted to use cannabis within 12 hours of a testing session and current smokers are not eligible to participate.

Study Design: This study will follow a double-blind crossover design. A crossover study means that the you will undergo treatment with a placebo (non-active drug) and an active medication (Atrovent®). There will be one testing period for each treatment separated by a washout period where no treatment will be given. The term double-blind means that both you and the researchers will not know which treatment is being received. In this case, the student researcher and Dr. Beth Davis will not know which treatment is being administered to you. However, Dr. Don Cockcroft will be unblinded from the beginning of the study so, if necessary, he will be able to identify which treatment you are taking.

Visit Schedule: You will be required to come to the Asthma Lab six times, three per treatment. These sessions will be approximately 30 minutes to 2 ½ hours long. At your first visit, information about your respiratory health, age, weight, height, allergies, medications, and general health will be collected. This will be followed with a baseline methacholine challenge test (MCT). If you are eligible, you will be given

Treatment 1 (placebo or Atrovent®) and 30 minutes later another MCT will be performed. You will be given an inhaler and be instructed on how to dose outside the lab. You will be required to return to the lab 7 days later for a final dose of Atrovent® and a post-dosing MCT. You will return your inhaler at this time and stop the treatment. Twenty four hours later you will need to return for a final MCT. Then you will enter a minimum seven day washout period where no treatment will be given. After seven days, you will return to the lab to begin Treatment 2 (i.e. repeat the same testing schedule as Treatment 1 but with the other treatment (placebo or Atrovent®). Below is a list of the instructions for each day of the study; approximate time requirements for the lab sessions are in bold.

Day 1: Health information and eligibility determined, consent sought, undergo baseline MCT, begin treatment #1 and undergo 30 minute post dose MCT (approximately 2 ½ hours)
Days 2-7: Take treatment as instructed (two puffs, three times a day)
Day 8: Return to lab for final dose and 30 minute post dose MCT (approximately 90 minutes)
Day 9: 24-hours after final dose return to lab for MCT (approximately 30 minutes)
Days 10-14: No treatment is given
Day 15: Return to lab, undergo baseline MCT, start Treatment #2 and undergo 30 minute post dose MCT (2 ½ hours)
Days 16-21: Take treatment as instructed (two puffs, three times a day)
Day 22: Return to lab for final dose and 30 minute post dose MCT (approximately 90 minutes)
Day 23: 24-hours after final dose return to lab for a final MCT (approximately 30 minutes)

If you forget to take a dose, take the dose as soon as you remember. For example, if it is your evening dose and you remember the next day, take four doses the next day – if you are not sure call, text or email Kayla @ email at kje889@usask.ca.

Study Procedures:

Spirometry: Baseline spirometry will be performed at each visit. This procedure measures your FEV_1 (the amount of air you can force out of your lungs in one second following a full inhalation) and FVC (forced vital capacity – the total amount of air you can exhale after a full inhalation). The procedure involves a full breath in, followed by exhalation as hard as you can for as long as you can through a mouthpiece that is attached to an nSpire KoKo spirometer (a hand-held device that is connected to a computer). This process will be repeated at least 3 times to achieve similar measurements of your lung function. Only FEV_1 or shortened spirometry maneuvers are required during the MCT. You will inhale fully and exhale forcefully as described above, but the exhalation time will be only 2-3 seconds.

Methacholine Challenge: MCT involves the inhalation of aerosolized methacholine, a substance known to cause airway smooth muscle contraction (i.e. bronchoconstriction) in people with asthma. Methacholine (MCh) is short-acting and is quickly metabolized by the body (i.e. broken down into inactive components). After completing initial spirometry as described above, you will inhale normal saline using normal breathing through a mouthpiece connected to a vibrating mesh nebulizer (Aerogen Solo nebulizer). You will breathe through the device until all the aerosolized saline solution has been inhaled (about 2 minutes). Shortened spirometry maneuvers (i.e. FEV_1) are then performed at 30 and 90-seconds post-inhalation. The next inhalation will be with methacholine and will begin 5 minutes after the start of the previous. You will again inhale the solution from the nebulizer using normal breathing until the solution is gone. Shortened spirometry maneuvers at 30 and 90 seconds post inhalation will be performed and the process will repeat every 5 minutes with increasing doubling doses of methacholine until your FEV_1 is reduced by at least 17%. Salbutamol (a rapid-acting bronchodilator) will be kept on hand in case

of an emergency (e.g. excessive airway narrowing) and will be provided to resolve any breathing difficulties prior to leaving the lab.

Responsibilities: As a participant, you will be expected to (1) follow the directions of the investigators and researchers and (2) report all medications (prescribed, over-the-counter and herbal products) being used. These steps are important both for your own safety as well as for accurate interpretation of the test results. The study-investigator may remove you from the study early in the event or non-compliance with the above responsibilities.

Benefits of Participating in This Study: If you choose to participate in this study, there will be no direct benefit for you personally. However, your participation will further the understanding of the usefulness of Atrovent® for the treatment of asthma.

Honorarium: If you choose to participate in this study, you will not be charged for any research related materials or procedures. Although you will not be paid for your participation, you will receive an honorarium in the amount of $270.00 ($25 per methacholine challenge and $5 per day for days dosing outside the lab.) The honorarium will cover any out of pocket expenses (e.g. parking, meals, transportation) and provide additional compensation for your time. If you enter the study and then decide to withdraw, we will compensate you for your time and expenses, proportionate to the amount of time you have been in the study. Honorariums totaling more than $100 are subject to declaration to Revenue Canada and for that purpose you will be required to supply your social insurance number.

Potential Risks and Discomforts: Methacholine Testing (MCT) may cause the following symptoms: wheezing (10%), coughing (25%), mild shortness of breath (21%), dizziness (6%) and/or headaches (2%). These effects do not last long and should go away without treatment. If you do respond to MCh, you will be given the option of receiving salbutamol following the MCh challenge to ensure complete and prompt recovery from any airway constriction or other symptoms. This is not an option after the first and last doses of each treatment however as these are the time points when the study inhalers are administered. Note however that if salbutamol is required for emergency purposes, deviations from the study protocol/design will occur. Excessive bronchoconstriction of an emergency nature following MCT is extremely rare.

Ipratropium bromide (Atrovent® HFA) consumer information indicates the following side effects may occur while taking this medication: headache; dizziness; nausea or feeling sick; digestive problems like constipation, diarrhea and vomiting; impaired voice sounds; throat irritation, cough, dry mouth or dry throat and bad taste. The frequency with which these potential adverse events occur is not provided. We do not anticipate any significant side effects to occur from the use (dose or duration) of ipratropium bromide in this study.

Unknown or unanticipated risks/side effects may occur and you should notify study staff of any side effect you are experiencing. If you feel the side effect is of an emergency nature – seek medical treatment immediately (i.e. go to a hospital emergency department) and inform study staff as soon as possible.

Voluntary Withdrawal: Your participation in this study is voluntary and as such, you may withdraw from the study at any time. No reason needs to be given for your withdrawal, and your current health care and/or academic status (if you are a student at the U of S) will not be impacted in any way. The data collected about you until the point of withdrawal may be retained for analysis. During the course of the study, you will be notified if any new information becomes available that may affect your willingness to participate.

Study-Related Injury: In the unlikely event of an adverse effect arising related to the study procedures, trained staff will be available throughout the conduct of the study who can respond immediately. Necessary medical treatment will be made available at no additional cost to you. By signing this document, you do not waive any of your legal rights against the investigators or anyone else.

Confidentiality: In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. Your name will not be attached to any information, nor mentioned in any study report, nor be made available to anyone except the research team (i.e. non-identifying codes will be used for your data e.g. IPT001). Any identifying information collected from you will remain under lock and key at the lab. Note that no guarantee can be made for complete confidentiality. For quality assurance and/or monitoring purposes, the University of Saskatchewan Biomedical Research Ethics Board reserves the right to inspect research records and medical records that may identify you in the presence of the investigator or his or her designate. It is the intention of the research team to publish results of this research in scientific journals and to present the findings at related conferences and workshops, but your identity will not be revealed.

Your study records will be kept for at least 5 years in a secured area. After storage, your study records will be shredded in a confidential manner.

Contact Regarding Any Questions/Concerns About The Study: If you have any questions or concerns regarding this study before or during participation, you may contact Kayla at kje889@usask.ca or Dr. Davis at

If you have any concerns about your rights as a research participant and/or your experiences while participating in this study, contact the Chair of the University of Saskatchewan Research Ethics Board, at The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Following the completion of the study, you may contact the study staff (i.e. student researcher at kje889@usask.ca if you're interested in obtaining a summary of the results and/or provision of any research article regarding the study when (if) available.)

UNIVERSITY OF SASKATCHEWAN

Consent:

Study Title: Regular use effect of inhaled ipratropium bromide on airway responsiveness to methacholine in well-controlled asthma.

- I have read the information in this consent form.
- I understand the purpose and procedures and the possible risks and benefits of the study.
- I was given sufficient time to think about it.
- I had the opportunity to ask questions and have received satisfactory answers.
- I understand that I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future health care, my academic standing or my employment.
- I give permission to the use and disclosure of my de-identified information collected for the research purposes described in this form.
- I understand that by signing this document I do not waive any of my legal rights.
- I will be given a signed copy of this consent form. □ provided or □ declined

I agree to participate in this study:

Printed name of participant: Phone number

Signature Date

Printed name of person obtaining consent:

Signature Date

Appendix E: BIO #1522 Recruitment Materials

Paws Announcement:

The Asthma Research Lab is conducting a research study to examine the effects of regular inhaled ipratropium bromide on airway responsiveness to methacholine in well-controlled asthma. Some eligibility criteria include:

- You are 18 years of age or older
- You are a non-smoker
- You have controlled mild to moderate asthma

Participation will require 6 visits to the asthma lab. Visits and testing can be done on any day of the week (including weekends).

This is a Physiology and Pharmacology BSc. Honours student project being conducted under the supervision of Dr. Don Cockcroft and Dr. Beth Davis.

You can check out the lab website and obtain a copy of the consent for under the "Studies Needing Participants" tab.

An honorarium will be provided to those individuals who meet all eligibility criteria and participate in the project.

For more information, contact the student researcher:

Kayla Cropper
kje889@usask.ca

Participants Needed!

UNIVERSITY OF SASKATCHEWAN
Fall 2019

Hello! You are receiving this because you have recently filled an inhaler at _____ Kayla Cropper, a Pharmacy assistant at your pharmacy is conducting a research study on an asthma medication. If you are interested, please see the details below:

If you are interested in participating, please contact Kayla at: **kje889@usask.ca**

Regular use effect of inhaled ipratropium bromide on airway responsiveness to methacholine in well controlled asthma:

Purpose of Study: This study is being conducted to determine if ipratropium bromide (e.g. Atrovent®) taken three times daily for one week (i.e. 2 puffs in the morning, 2 puffs in the afternoon and 2 puffs in the evening for 7 days) reduces the effectiveness of this medication. **Background Information:** Atrovent® belongs to a class of medications called bronchodilators. Bronchodilators, which include Atrovent® and salbutamol (Ventolin®), are often referred to as rescue medication and are used to quickly relieve wheezing and shortness of breath in asthma. Bronchodilators also offer bronchoprotection against methacholine induced airway smooth muscle contraction. Methacholine testing is used clinically to help determine if airway hyperresponsiveness is present and assists in diagnosing asthma. Previous studies have demonstrated that bronchodilators acting on beta receptors (e.g. salbutamol and salmeterol) become less effective with frequent use. Very little is known regarding the effectiveness of Atrovent® following regular use. **Eligible Participants (12-15 required):** To participate in this study, you must be at least 18 years old. Criteria for your enrollment includes appropriate values for FEV_1 and PD_{20}. These values will be measured upon your first lab visit. You may not participate if you are pregnant, nursing, or suffer from cardiovascular problems. Please disclose if you are on any medications or have any pre-existing health conditions. Certain medications and health conditions may affect results of this study or may put your health at risk if you choose to participate. The study will require that you have not used Atrovent®, or similar acting medication, in the past 30 days. You will be allowed to use a salbutamol inhaler as needed; however, it cannot be used within 6 hours of lab visits. You are not permitted to use cannabis within 12 hours of a testing session and current smokers are not eligible to participate. If you choose to participate in this study, you will not be charged for any research related materials or procedures. You will not be paid for your participation. You will receive an honorarium in the amount of $260.00 ($25 per methacholine challenge and $5 per day for days dosing outside the lab.) If you do not meet criteria upon test completion you will receive $25. The honorarium will cover any out of pocket expenses and provide additional compensation for your time.

Appendix F: BIO #1959 Certificate of Approval

Biomedical Research Ethics Board (Bio-REB) 19/Jun/2020

Certificate of Approval

Application ID:	1959
Principal Investigator:	Donald Cockcroft Department: Department of Medicine
Locations Where Research Activities are Conducted:	Room 346 Ellis Hall Asthma Research Lab, Canada
Student(s):	
Funder(s):	College of Medicine Department of Medicine
Sponsor:	University of Saskatchewan
Title:	Tiotropium Efficacy Against Allergen Induced Early Asthmatic Responses
Protocol Number:	
Approved On:	18/Jun/2020
Expiry Date:	17/Jun/2021
Approval Of:	* Notice of Ethical Review Response, rec'd 15-Jun-2020 * Biomedical Application, Prospective, rec;d 15-Jun-2020 * Participant Information and Consent Form, Version June 15, 2020 * Study Protocol, Version 1.0
Acknowledgment Of:	
Review Type:	Full Board
Meeting Date:	20/May/2020
IRB Registration Number:	Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal risk projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Appendix G: BIO #1959 Health Canada No Objection Letter

Health Canada / Santé Canada

Therapeutic Products Directorate
5th Floor, Holland Cross, Tower B
Address Locator # 3105A
OTTAWA, Ontario
K1A 0K9

07 October 2020

Beth Davis
Research Scientist/Co-Investigator
University of Saskatchewan
Room 350 Ellis Hall, 103 Hospital Drive
SASKATOON, Saskatchewan
S7N 0W8

Your file *Votre référence*
HC6-24-c241380
Our file *Notre référence*

No Objection Letter RE: Protocol # UOFSBIOREB#1959 (Version 3.0)

Dear Beth Davis:

I am pleased to inform you that the information and material to support your Clinical Trial Application for **TIOTROPIUM BROMIDE/ ALLERGENIC EXTRACT – NON POLLEN/ ALLERGENIC EXTRACT POLLENS/ ALLERGENIC EXTRACT MIXTURE OF STANDARDIZED MITES/ ALLERGENIC EXTRACT STANDARDIZED MITE DF/ ALLERGENIC EXTRACT STANDARDIZED MITE DP/ STANDARDIZED CAT HAIR AP/ STANDARDIZED GRASS POLLEN-TIMOTHY**, control number **241380**, received on August 27, 2020, have been reviewed and we have no objection to your proposed study. I would remind you of the necessity of complying with the *Food and Drug Regulations,* Division 5, in the sale of this product for clinical testing. In addition, the regulations impose record keeping responsibilities on those conducting clinical trials. You are also reminded that all clinical trials should be conducted in compliance with the Therapeutic Products Directorate's *Guideline for Good Clinical Practice.*

Please note that Health Canada has implemented electronic reporting of adverse drug reactions and is currently in pilots with some sponsors. Those sponsors who have an established electronic connection with Canada Vigilance Production stream should submit their reports using the distribution rules provided to them by Health Canada, and reporting to multiple directorates is no longer required. For the sponsors who have not yet established this connection, they should continue submitting their reports to the applicable directorate by fax or by courier. The following website provides further clarification on Health Canada's adverse drug reactions reporting requirements for clinical trials: https://www.canada.ca/en/health-canada/services/drugs-health-products/drug-products/health-canada-clinical-trials-database.htm

Consistent with Health Canada's Notice - *Registration and Disclosure of Clinical Trial Information* of November 30, 2007, sponsors are encouraged to register their clinical trials within 21 days of the trial's onset, using a publicly available registry that conforms with international standards for registries such as: Clinicaltrials.gov (www.clinicaltrials.gov); Current Controlled Trials (www.controlled-trials.com).

Should you have any questions concerning this letter, please contact the Office of Clinical Trials (613) 941-2132.

Yours sincerely,

LL/mw

Canada

Appendix H: BIO #1959 Certificate of Approval Amendments

UNIVERSITY OF SASKATCHEWAN

Biomedical Research Ethics Board (Bio-REB) 04-Nov-2020

Certificate of Approval Amendment

Application ID: 1959

Principal Investigator: Donald Cockcroft Department: Department of Medicine

Locations Where Research Activities are Conducted: Room 346 Ellis Hall Asthma Research Lab, Canada

Student(s): Kayla Cropper

Funder(s): College of Medicine
Department of Medicine

Sponsor: University of Saskatchewan

Title: Tiotropium Efficacy Against Allergen Induced Early Asthmatic Responses

Protocol Number:

Approved On: 04/11/2020

Expiry Date: 17/06/2021

Approval Of:
* TIO AC Revised Protocol Per HC IR#1 CLEAN
* TIO AC CF IR#1 Response CLEAN October 5, 2020
* Response to Information Request #1 (IR#1) to Health Canada
* Health Canada No Objection Letter

Acknowledgment Of:
* TIO AC Revised Protocol Per HC IR#1 TRACKED
* TIO AC CF IR#1 TRACKED Response October 5, 2020
* Response to Information Request #1 (IR#1) to Health Canada
* Health Canada No Objection Letter
* UnivRS ID 1959 IR#1 Amendment Form October 2020
* Reviewed with COVID-19 safety considerations in mind

Review Type: Delegated Review

IRB Registration Number: Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

UNIVERSITY OF SASKATCHEWAN

Certificate of Approval Amendment

Application ID: 1959

Principal Investigator: Donald Cockcroft Department: Department of Medicine

Locations Where Research Activities are Conducted: Room 346 Ellis Hall Asthma Research Lab, Canada

Student(s): Kayla Cropper

Funder(s): College of Medicine
Department of Medicine

Sponsor: University of Saskatchewan

Title: Tiotropium Efficacy Against Allergen Induced Early Asthmatic Responses

Protocol Number:

Approved On: 17-Dec-2020

Expiry Date: 17-Jun-2021

Approval Of:
* Covid-19 Precautions & Info_Version 1 rec'd 15-Dec-2020
* Covid-19 Risk Form_rec'd 15-Dec-2020

Acknowledgment Of:
* BIO 1959 REB Amendment
* Bio 1959 Amendment NER_rec'd 15-Dec-2020
* Reviewed with COVID-19 safety precautions in mind

Review Type: Delegated Review

IRB Registration Number: Not Applicable

CERTIFICATION

The University of Saskatchewan Biomedical Research Ethics Board (Bio-REB) has reviewed the above-named project.The project is acceptable on scientific and ethical grounds. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to this project, and for ensuring that the authorized project is carried out according to governing law. This approval is valid for the specified period provided there is no change to the approved project.

FIRST TIME REVIEW AND CONTINUING APPROVAL

The University of Saskatchewan Research Ethics Boards review above minimal projects at a full-board (face-to-face) meeting. If a project has been reviewed at a full board meeting, a subsequent project of the same protocol may be reviewed through the delegated review process. Any research classified as minimal risk is reviewed through the delegated (subcommittee) review process. The initial Certificate of Approval includes the approval period the REB has assigned to a study. The Status Report form must be submitted within one month prior to the assigned expiry date. The researcher shall indicate to the REB any specific requirements of the sponsoring organizations (e.g. requirement for full-board review and approval) for the continuing review process deemed necessary for that project.

REB ATTESTATION

In respect to clinical trials, the University of Saskatchewan Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Part 4 of the Natural Health Products Regulations and Part C Division 5 of the Food and Drug Regulations and carries out its functions in a manner consistent with Good Clinical Practices. Members of the Bio-REB who are named as investigators, do not participate in the discussion related to, nor vote on such studies when presented to the Bio-REB. This approval and the views of this REB have been documented in writing. The University of Saskatchewan Biomedical Research Ethics Board is constituted and operates in accordance with the current version of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2 2018).

Appendix I: BIO #1959 Participant Consent Form

UNIVERSITY OF SASKATCHEWAN

Participant Information and Consent Form

Title of Study:	Tiotropium efficacy against allergen induced early asthmatic responses
Protocol #:	UofS BIO REB #1959
Principal Investigator:	Dr. Donald Cockcroft, MD, FRC(P)C Department of Medicine Division of Respirology, Critical Care and Sleep Medicine University of Saskatchewan
Co-Principal Investigator:	Dr. Beth Davis, PhD Department of Medicine Division of Respirology, Critical Care and Sleep Medicine University of Saskatchewan
24-hour Emergency Contact:	**Dr. Beth Davis** **beth.davis@usask.ca (email)**

Introduction:

You are invited to take part in this clinical research study because you have been diagnosed with allergic asthma and you are between 18 and 65 years of age.

Your participation is entirely voluntary. If you decide to participate, you are still able to withdraw at any time. If you choose not to participate or choose to withdraw from the study, your access to health care, your employment and/or your academic status will not be affected.

Please take time to read the following information carefully. You may ask the study staff any questions you may have before, during and after the study. Please feel free to discuss this study with your family, friends and/or primary care physician before making a decision about participating.

Funding:

The University of Saskatchewan (USask) Department of Medicine has provided funding to conduct this clinical trial. The Investigators have no financial interest in the outcome of this study. The Investigators

are not being paid beyond their regular salary to conduct this clinical trial. The study drug will be obtained from the manufacturer (Boehringer Ingelheim Canada).

Purpose of the Study:

Tiotropium (Spiriva Respimat®) is a medication used to treat respiratory conditions. Technically, tiotropium is referred to as a long acting muscarinic antagonist or "LAMA". The term long acting suggests the drug is used once a day. Muscarinic refers to a type of receptor on airway smooth muscle involved in causing bronchoconstriction or a tightening of the airway which makes breathing more difficult. Antagonist means it blocks or inhibits the effect. Therefore, tiotropium blocks muscarinic receptors on airway smooth muscle and prevents bronchoconstriction. Tiotropium was recently approved by Health Canada for use as add-on therapy in poorly controlled asthmatics. Although tiotropium is approved for use as an add-on therapy in poorly controlled asthma, the use of tiotropium is not approved for use in mild allergic asthma. The use of tiotropium in this study is therefore investigational or experimental. Health Canada has approved the use of tiotropium in this study.

Recent research suggests tiotropium may also have anti-inflammatory effects. The potential anti-inflammatory effect of tiotropium is relevant to individuals with allergic asthma since allergen exposure not only causes acute bronchoconstriction (i.e. asthma symptoms) but also leads to airway inflammation. The purpose of this study is to investigate the protective effects of tiotropium on the response to allergen exposure in individuals with mild allergic asthma.

Study Design Overview:

This study is designed as a single-center, double-blind, randomized, placebo-controlled, cross-over study. There are two treatments, tiotropium and matching placebo. Single-center means only researchers from the U of S Asthma Research Lab are conducting the study. Double-blind means neither you nor the Investigators will know which treatment you are receiving when but this information is available in an emergency situation. Randomized means you will receive the treatments in random order. Placebo-controlled means one of the two treatments will be an inactive treatment (i.e. no active drug ingredient). Cross-over means you will receive both treatments but not at the same time. The Investigators plan to collect data from 15 individuals with mild allergic asthma.

Eligibility Criteria:

You are eligible to participate in this study if you are in general good health with no other medical conditions or lifestyle activities that would potentially alter the outcome of study procedures. You must be at least 18 years of age but not older than 65. You must have been diagnosed with asthma at least 3 months ago. Your asthma must be stable or well-controlled. Your baseline lung function, measured as the amount of air that you can forcefully exhale in the first second of exhalation (FEV_1), must be $\geq$87 % of what is predicted for a person of your age, height, and sex. You must have a positive skin prick test to one of the study allergens and you must demonstrate airway responsiveness to methacholine (i.e. respond with a decrease in lung function of at least 20% following inhalation of methacholine).

You may not participate in this study if you require medications other than occasional salbutamol (e.g. Ventolin®) to control your asthma or anti-histamines to treat allergy symptoms. Salbutamol use may not exceed 4 times per week and anti-histamine use will need to be withheld prior to study visits. The duration to withhold the medication will depend on the anti-histamine used and this will be discussed with you.

You will be required to withhold salbutamol for six hours prior to any lab visit. However, should you need to use salbutamol or an anti-histamine within the required washout timeframe, you should take the medication and then contact study personnel to reschedule your visit, which may mean repeating previous visits.

You will not be eligible to participate if any of the following apply:

- you are pregnant or breast-feeding
- you have another lung condition or medical condition that would put you at risk if you participate
- you are a current smoker of nicotine products (e.g. cigarettes) or you have a significant smoking history (e.g. pack a day for 10 years)
- you use cannabis or other inhaled recreational products (e.g. e-cigarettes or other vaping products) on a daily basis; occasional use will require a 24 hour withhold
- you have experienced a worsening of your asthma within 4 weeks of starting this study that required a change in how you treat your asthma (e.g. use of inhaled corticosteroids)
- you have been exposed to a trigger that worsens your asthma (e.g. cat) within the last 4 weeks
- you have experienced a respiratory infection within the last 4 weeks
- you have a diagnosis of narrow angle glaucoma
- you have a diagnosis of urinary retention
- you have a known hypersensitivity to tiotropium or components of tiotropium formulation (e.g. benzalkonium chloride)
- you have a history of anaphylaxis or angioedema
- you currently use any of the following medications:
    - inhaled corticosteroid including combination therapies
    - inhaled muscarinic antagonists – except study treatment (e.g. ipratropium bromide)
    - long-acting beta$_2$-agonists (LABA; e.g. formoterol)
    - leukotriene receptor antagonists (e.g. montelukast)
    - biologics (e.g. benralizumab)
    - allergen immunotherapy
    - mast cell stabilizers (e.g. nedocromil sodium)

Visits, Procedures and Time Commitment:

If you decide to take part in this study, you will be required to attend the research lab (Room 346 Ellis Hall) on four occasions over the course of approximately 4 weeks. The study visits and procedures are summarized in Table 1 on page 4 of this document. At Visit 1, which will require about 2.5 hours of your time, study staff will provide an overview of the study purpose and procedures and answer any questions you may have. If you choose to participate, you will be required to sign the consent form (i.e. this document). After consent is obtained, you will be required to undergo the following tests to determine your eligibility and to capture baseline measurements: fractional exhaled nitric oxide (FeNO), spirometry, methacholine challenge, sputum induction, skin prick testing and skin titration endpoint testing. If you are eligible, you will be randomly assigned treatment 1. If you do not meet spirometry or methacholine

challenge eligibility requirements at first attempt, one re-screen attempt will be allowed. You will be trained on the use of the inhaler and the first dose will be administered. You will self-administer daily doses (2 puffs once a day) for the next six days before returning to the lab for Visit 2.

At Visit 2, which will require about 6 hours of your time, study staff will ask you about daily dosing and a final dose will be administered. Thirty minutes after dosing, blood pressure, FeNO and baseline spirometry will be performed followed by allergen inhalation challenge and then sputum induction. After Visit 2, there will be a washout period of at least two weeks before you return to the lab for Visit 3 and the start of treatment 2. The term "washout period" means you will not be receiving any treatment during this time.

Visit 3 procedures are similar to Visit 1 procedures and will require about 2 hours of your time. Procedures at this visit include baseline measurements of FeNO, spirometry, methacholine challenge and sputum induction. Following collection of these data, blinded treatment 2 will be provided to you and the first dose will be administered. You will again self-administer daily doses for the next six days prior to returning to the lab for Visit 4.

Visit 4 procedures are identical to Visit 2 procedures and will again require about 6 hours of your time.

Table 1 – Visits, procedures and time commitment:

Visit 1	Treatment Period	Visit 2	Washout	Visit 3	Treatment Period	Visit 4
Consent FeNO Spirometry MCT Sputum SPT STE Titration Randomization First Dose Tx1	Self administer Treatment 1 for 6 days	Final Dose Tx1 Blood pressure FeNO (pre AC) Spirometry Allergen Challenge FeNO (5 hrs post AC) Sputum (5 hrs post AC)	Minimum 2 weeks	FeNO Spirometry MCT Sputum Crossover Treatment First Dose Tx2	Self administer Treatment 2 for 6 days	Final Dose Tx2 Blood pressure FeNO (pre AC) Spirometry Allergen Challenge FeNO (5 hrs post AC) Sputum (5 hrs post AC)
Approx. time 2.5 hours		Approx.. time 6 hours		Approx. time 2 hours		Approx. time 6 hours

FeNO = fractional exhaled nitric oxide; MCT=methacholine challenge; SPT = skin prick test; STE = skin test endpoint; AC = allergen challenge

Study Assessments

Fractional Exhaled Nitric Oxide

The level of nitric oxide in your exhaled breath is an indicator of airway inflammation. The test requires you to inhale and exhale fully into a handheld device via a filter mouthpiece. The handheld device is connected to a small machine that measures the amount of nitric oxide in your exhaled breath and displays the value on the screen of the machine. At least two tests will be performed each time FeNO is measured. FeNO will be measured at all visits, twice on allergen challenge visits.

Spirometry

Spirometry is a necessary component of bronchoprovocation testing. Spirometry will be performed according to current industry standards and used to assess your resting lung function for determining eligibility criteria and for monitoring the magnitude of airway narrowing induced during allergen and methacholine challenges. To perform spirometry, you will be required to breathe through a mouthpiece with your nose clipped. You will inhale as much air as you can and then exhale forcefully until your lungs are empty. The spirometer is connected to a computer and the computer software generates various lung function parameters. The parameters we are interested in are the forced expiratory volume during the first second of exhalation (FEV_1) and the forced vital capacity (FVC). More simply put, FEV_1 is the amount of air you are able to forcefully exhale in one second and the FVC is the total amount of air you are able to forcefully exhale after fully inhaling. Spirometry is performed at all visits, multiple times.

Skin Prick Testing

Skin prick testing will be used to determine what allergies you have and identify the allergen to be used in the allergen challenge. Drops of allergen extracts (including a positive and a negative control) will be placed on your forearm and introduced through the skin by pricking with a lancet. After 10-15 minutes, the skin reaction will then be evaluated based on size. The allergen chosen for your allergen challenges will depend on the largest skin reaction produced through skin prick testing as well as on your clinical history regarding exposure to that particular allergen. Skin prick testing will be performed once at Visit 1.

Skin Test Endpoint (STE) Titration

The STE Titration test is similar to skin prick testing but instead of applying several different allergen extracts, duplicate drops of increasing concentrations of the specific allergen chosen from the skin prick test will be applied to the forearm, pricked with a lancet, and assessed to determine the concentration that produces a particular sized skin reaction (i.e. reaction resembling a mosquito bite that is less than 2mm in diameter). Skin test endpoint testing is performed once at Visit 1.

Methacholine Challenge Testing (MCT)

MCT will be performed with the Aerogen Solo® vibrating mesh nebulizer per an established standardized procedure known as the volumetric method. Baseline spirometry will first be performed to obtain at least two reproducible measurements of your resting FEV_1. Next, you will wear nose clips and inhale aerosolized saline through a mouthpiece while breathing normally until the nebulizer is empty

(approximately 1.5-2.5 minutes). FEV_1 measurements will be recorded at 30 and 90 seconds post-inhalation, and the next inhalation will begin five minutes after the start of the previous inhalation. Each subsequent inhalation will entail doubling doses of methacholine, a compound that may cause your airways to narrow (i.e. make it harder to breathe). The procedure will continue until your FEV_1 has dropped at least 17% from the value obtained after inhaling saline. You will be required to undergo two methacholine challenge tests, one at Visit 1 and one at Visit 3.

Allergen Challenge Testing

The early asthmatic response (EAR) allergen challenge will be performed per standard method. Following baseline spirometry, you will inhale doubling doses of allergen like was done with methacholine. Two FEV_1 measurements, one minute apart, will be recorded ten minutes after you finish inhaling each dose. Twelve minutes will elapse between the start of one inhalation to the start of the next inhalation. The allergen challenge ends when your FEV_1 drops at least 20% from the highest measurement obtained during baseline spirometry. You will be required to remain in the lab for the next 5 hours and perform FEV_1 measurements at various time points. FeNO will be measured again at 5 hours post allergen inhalation just prior to the final spirometry measurement. You will then self-administer 200mcg salbutamol and undergo a ten minute wait period before the process of sputum induction commences. Following sputum induction, you will self-administer an additional 200mcg salbutamol and 500mcg fluticasone propionate (or equivalent). A final spirometry measurement will be obtained to ensure lung function has returned to a safe level prior to leaving the lab. You will undergo two allergen challenges, one at Visit 2 and one at Visit 4.

Sputum Induction

Airway secretions (i.e. mucous or sputum) collected from your lungs are valuable samples for use in assessing airway inflammation. To help you produce sputum, you will be asked to inhale 3 different concentrations (3%, 5% and 7%) of "salty" water known as hypertonic saline, each for 7 minutes. Inhaling hypertonic saline creates an environment in your airways that draws sputum from your lungs into the airways where it can be expectorated (i.e. coughed, hacked, huffed up). After each inhalation you will be asked to blow your nose and rinse your mouth with water before trying to produce a sample. After each inhalation you will also perform an FEV_1 measurement to ensure your lung function is stable. You will undergo the process of sputum induction once at each visit.

Optional Sub-study

Sputum samples will be used to produce a layer of cells from your airway on microscope slides. These slides are then used to count the number of inflammatory cells on the slides which provides information about airway inflammation (i.e. the type of cells in the airway) and how the inflammation changes with exposure to allergen and following treatment with tiotropium. These data are required for the current study.

Sputum samples can also be used to look at mediators or biomarkers involved in different cellular processes or pathways that are activated by allergen exposure or inhibited by treatment. Although this is not the purpose of the current study, we would like to store part of your sputum sample for future research on mechanisms of airway inflammation specific to allergic asthma and how tiotropium may affect these events. Your samples will not contain identifiable information. Your samples will be stored in a freezer in the Asthma Research Lab for a period not exceeding 15 years. Your samples will not be shared. Any

future research conducted on your samples will be reviewed and approved by the University of Saskatchewan Biomedical Research Ethics Board but your consent will not be sought.

If you change your mind about allowing your samples to be stored, contact the Investigators and let them know you would like your samples destroyed. Your samples will be removed from the freezer and destroyed. Any data already collected from the use of your samples will be retained, however no additional future research will be performed.

You will be able to indicate your willingness (or not) to store your sputum samples for future use by indicating yes or no on the signature page of this document.

Potential Risks and Discomforts:

Fractional exhaled nitric oxide

There are no known risks associated with FeNO testing in individuals with mild allergic asthma.

Spirometry

There are no known risks associated with performing spirometry in individuals with mild allergic asthma.

Skin prick testing and skin titration endpoint testing

Skin prick testing and skin titration endpoint testing may cause itching and swelling where the allergens were administered. This usually subsides quickly. If symptoms persist, an antihistamine, topical corticosteroid cream, and/or an ice pack may be used to treat the reaction. Severe reactions from skin prick testing are very rare but may cause anaphylaxis which can be fatal. Such severe reactions typically occur shortly after testing during which time study staff will be monitoring you and ready to treat such a reaction immediately.

Methacholine Challenge Testing

Adverse reactions associated with inhaling methacholine may include headache, throat irritation, light-headedness and itching. Additionally, chest tightness, cough or wheezing may occur. Adverse reactions are generally mild and quickly resolve without the use of treatment. Lung function is monitored throughout the test and bronchodilator (salbutamol/Ventolin®) is immediately available if necessary. Severe adverse reactions are extremely rare.

Allergen Challenge Testing

Allergen inhalation challenges are relatively safe when performed in centers with highly experienced personnel and with proper oversight. Allergen challenge testing may cause coughing, chest tightness, wheezing, and difficulty breathing. The testing method is designed to minimize the risk of causing a severe reaction but there is a risk of severe airway narrowing (i.e. anaphylaxis) developing. If such a situation occurs, you will be treated immediately. Symptoms induced by the allergen challenge usually resolve over time without treatment or may be reversed by use of a bronchodilator (e.g. Ventolin®) and/or inhaled corticosteroid (e.g. Pulmicort®). Both of these treatments will be provided to you prior to leaving the lab.

The use of allergen extracts for inhalation purposes is not approved by Health Canada, however, Health Canada has authorized the use of allergen extracts for inhalation purposes in this research study.

Sputum Induction

Inhalation of hypertonic saline may cause bronchoconstriction but this is not anticipated in individuals with mild allergic asthma. As a precaution, salbutamol (Ventolin®) is administered prior to undergoing sputum induction. In the event that severe bronchoconstriction occurs during the process of sputum induction, you will be treated with salbutamol (Ventolin®). In addition, FEV_1 monitoring is performed during the process to monitor and detect any bronchoconstriction.

Tiotropium treatment

In the proposed study population (i.e. mild allergic asthma) adverse events associated with taking tiotropium are not anticipated. In theory, based on how the drug works, dry mouth may develop. The product monograph for tiotroium indicates adverse events occurred in 1.4-3.3% of individuals following use of tiotropium. Events included oral thrush (infection at back of mouth), dry mouth, gastroesophageal reflux, sinusitis (inflamed sinuses), cough, dysphonia (difficulty speaking) and rash. Less common events (occurring in <1%) were also observed and include: atrial fibrillation (abnormal heart beat), supraventricular tachycardia (increased heart rate), blurred vision, gingivitis (swelling of gums), joint swelling, dizziness, epistaxis (nose bleed), laryngitis and dry skin. These studies were conducted in individuals with severe asthma who were using additional medications such as inhaled corticosteroids and long acting beta agonists. Use of these types of medications are associated with some of the reported adverse events (e.g. oral thrush) In addition, these individuals were treated with tiotropium for a much longer duration (12 weeks to one year).

Placebo treatment

There are no anticipated adverse events associated with receiving placebo treatment.

Responsibilities of the Participant:

As a study participant, you will be expected to:

a. Follow the directions of the study staff
b. Report all medications (prescribed, over-the-counter, herbal etc.) currently being used
c. Report any changes in your health or medications used during the study

The study investigator may remove you from the study early for non-compliance with the above responsibilities.

Your health and wellbeing are most important and so, if faced with a situation where you must use a medication that will (or may) interfere with the study, we ask that you take the medication and contact us to reschedule your appointment.

Benefits of Participating in this study:

If you choose to participate in this study, there will be no direct benefit to you. This study will provide information on whether or not tiotropium is useful for treating allergic asthma.

Voluntary Withdrawal:

Participation in this study is entirely voluntary and you have the right to withdraw from the study at any time without providing a reason. The decision to withdraw will not affect your future health care, employment or academic standing. Data collected from you before your withdrawal may still be analyzed. You will be promptly notified if any new information regarding any aspect of this study becomes available that may affect your willingness to continue your participation.

Study-Related Injury:

In the unlikely event of a medical emergency seek immediate medical attention and notify study staff as soon as possible. Necessary medical treatment will be made available to you at no cost. By signing this document, you do not waive any of your legal rights against the investigators or anyone else.

Honorarium:

If you choose to participate in this study, you will not be charged for the study drugs or any research-related procedures. You will not be paid for your participation; however, you will be provided with an honorarium in the amount of $600 in order to cover your time and out-of-pocket expenses. In the event all study visits are not completed, the honorarium will be prorated based on the study procedures/visits you were able to complete. The University of Saskatchewan will require your social insurance number (asked for on the signature page of this form) to provide the honorarium and a T4A will be sent to you at the appropriate time.

Confidentiality:

In Saskatchewan, the Health Information Protection Act (HIPA) defines how the privacy of your personal health information must be maintained so that your privacy will be respected. Your name will not be attached to any information or mentioned in any study report or made available to anyone except the study staff and personnel of the University of Saskatchewan responsible for issuing the honorarium. Study records will identify you by a study code such as TIOAC01. All study related information will be stored in a locked room (e.g. asthma lab or investigator office) but no guarantee of complete confidentiality can be made. For quality assurance and/or monitoring reasons, the University of Saskatchewan Biomedical Research Ethics Board and/or Health Canada representatives have the right to inspect research records in the presence of the principal investigator or designate and some records may contain identifying information. It is the intention of the Investigators to publish results of this research in scientific journals and to present the findings at related conferences, but your identity will not be revealed. Per Health Canada guidelines, your records will be stored for a period not less than 25 years. After storage, your records will be shredded in a confidential manner.

Public Registry Listing and Study Results:

A description of this clinical trial will be posted on http://www.clinicaltrials.gov, as required by Health Canada. This website will not include information that can identify you. You can search this website at any time.

Study results and your individual results will be available to you once all study data collection is complete. If you are interested in receiving a copy of the results, please check the appropriate box(es) on the last page of this document.

Questions or Concerns Regarding the Study:

If you have any questions or concerns or would like additional information about this study before or during participation, you may contact Dr. Davis at or Dr. Cockcroft at

If you have any concerns about your rights as a research participant and/or your experiences while participating in this study, contact the Chair of the University of Saskatchewan Research Ethics Board at The Research Ethics Board is a group of individuals (scientists, physicians, ethicists, lawyers and members of the community) that provide an independent review of human research studies. This study has been reviewed and approved on ethical grounds by the University of Saskatchewan Research Ethics Board.

Consent:

Study title: Tiotropium efficacy against allergen induced early asthmatic responses.

- I have read the information in this consent form.
- I understand the purpose, procedure and possible risks of the study.
- I was given sufficient time to think about it.
- I have had the opportunity to ask questions and have received satisfactory answers.
- I am free to withdraw from this study at any time for any reason and the decision to stop taking part will not affect my future medical care, employment or academic status.
- I agree to follow the instructions of the study staff and will immediately tell the study staff if I feel I have had any unexpected or unusual symptoms.
- I give permission for the use and disclosure of my de-identified personal health information collected for the research purposes described in this form.
- I understand that by signing this document, I do not waive any of my legal rights.
- I will be given a signed and dated copy of this consent form.

I consent to the storage of my sputum sample for future research on airway inflammation and how tiotropium alters airway inflammation specific to allergic asthma . □ YES or □ NO

I am interested in receiving a summary of group study results: □ YES or □ NO

I am interested in receiving my own study results: □ YES or □ NO

I agree to participate in this study:

Printed Name of Participant	Date
Signature of Participant	SIN #
Printed Name of Person Obtaining Consent	Date
Signature of Person Obtaining Consent	

Appendix J: BIO #1959 COVID-19 Forms

Informal COVID-19 Risk Disclosure

We ask that you voluntarily fill out this informal COVID-19 Risk Form so that lab members can be informed of your associated risk with being in the lab. Please use the highlight function to fill out and return back to us prior to your lab visit. If you are not comfortable with this, please let us know. If you would like to disclose this information verbally, we can arrange a phone call prior to your visit to go through the questions.

This is not an official form or binding by any means. However, it allows lab members to take into consideration any safety risks associated with lab visits during the pandemic. The aim of any information disclosed is only to keep all other lab members and participants safe during this time. The information you provide will only be available to the lab members: Kayla Cropper, Dr. Beth Davis and Dr. Donald Cockcroft and will not be used for any other purposes. Please do not put your name on this form. This form will be deleted after the study has been completed.

We ask that you let us know if anything changes regarding your COVID-19 exposure and that you remain open and honest about any of your COVID-19 exposure risks.

Part 1: Standard COVID-19 Screening from the Government of Canada

1. **Are you experiencing any of the following? If yes, please highlight which of the symptoms:**

- Severe difficulty breathing (e.g. struggling to breathe or speaking in single words)
- Severe chest pain
- Having a very hard time waking up
- Feeling confused
- Losing consciousness
- Mild to moderate shortness of breath
- Inability to lie down because of difficulty breathing
- Chronic health conditions that you are having difficulty managing because of difficulty breathing
- new or worsening cough
- shortness of breath or difficulty breathing
- temperature equal to or over 38°C
- feeling feverish
- chills
- fatigue or weakness
- muscle or body aches
- new loss of smell or taste
- headache
- gastrointestinal symptoms (abdominal pain, diarrhea, vomiting)
- feeling very unwell

A **close contact** is defined as a person who:

Provided care for the individual, including healthcare workers, family members or other caregivers, or who had other similar close physical contact without consistent and appropriate use of personal protective equipment; or

Lived with or otherwise had close prolonged contact (within 2 metres) with the person while they were infectious; or

Had direct contact with infectious bodily fluids of the person (e.g. was coughed or sneezed on) while not wearing recommended personal protective equipment.

Yes No

2. **Have you travelled to any countries outside Canada (including the United States) within the last 14 days?**

   Yes No

3. **Within the last 14 days did you provide care or have close contact with a symptomatic person known or suspected to have COVID-19?**

   Yes No

4. **Did you have close contact with a person who travelled outside of Canada in the last 14 days who has become ill**

   Yes No

Part 2: Saskatchewan and Saskatoon-Specific Questions

1. **Do you work outside the home?** Yes No

   a. If yes, do you come in close contact with co-workers? Yes No

   b. If yes, do you use personal protective equipment (mask, gloves etc.)? Yes No

2. **Have you visited any restaurants in the past month?** Yes No

3. **Have you visited any bars, pubs, or nightclubs in the past month?**

   Yes No

4. **Have you visited any public gyms in the past month?** Yes No

5. **When you go in public, how often do you wear a mask?**

   Always Often/Frequently Sometimes Never

6. **Please estimate the size of your bubble/close contacts:** _____
   ie) roommates, family members, friends etc. who you do not distance or wear masks around

7. **Do you have any personal contacts related to the school system?**
   ie) A close contact working in or attending schools.

Yes No

8. **Have you travelled outside of Saskatoon recently?** Yes No

9. **Have you travelled outside of Saskatchewan recently?** Yes No

10. **Please review the lists of businesses with potential COVID-19 exposures and outbreaks via the following links:**

    Outbreaks:
    https://www.saskatchewan.ca/government/health-care-administration-and-provider-resources/treatment-procedures-and-guidelines/emerging-public-health-issues/2019-novel-coronavirus/latest-updates/covid-19-active-outbreaks

    Daily exposure advisories:
    https://www.saskatchewan.ca/government/health-care-administration-and-provider-resources/treatment-procedures-and-guidelines/emerging-public-health-issues/2019-novel-coronavirus/latest-updates#advisories-events-locations

    a. Have you attended any of the businesses with potential exposures on the exposure day(s) indicated?

       Yes No

    b. Have you attended any of the locations with outbreaks on the exposure day(s) indicated?

       Yes No

Thank-you for voluntarily disclosing information about your COVID-19 exposure risks to the lab members in the Asthma Lab. If you have any questions, please let us know. If there are concerns over your potential exposure to COVID-19, we may reschedule your appointment to a later date to minimize risk of COVID-19 spread in the Asthma Lab.

Version 1

COVID-19 Precautions and Information:

Due to the rapidly evolving situation, Kayla has put together a form on everything you may want to know or need to know regarding COVID-19 and Asthma Lab visits. This is not an official document. This informal document is intended to help you understand how the lab will function during the pandemic to our current knowledge. The details provided on this form are subject to change as the situation changes. You will be notified of any changes to the best of our ability.

1. Entrance into the hospital:
   - Visitor restrictions have been placed on RUH including Ellis Hall.
   - To enter the building, you may need to undergo screening questions, a temperature check, you require a mask and may need to sanitize your hands depending on the specific requirements during the time of your visit.
   - Entrances that you can enter at include:
     - If you are driving:
       - **The Parkade Entrance at level 2**: (accessible from Hospital Drive)
         - You may park on P3, P4 or P5 – go down the stairs to RUH entrance
         - Parking on LP2 – go up the stairs until you reach RUH entrance
       - **JPCH Entrance at level 5:** (accessible from Hospital or Campus Drive)
         - You may park on P3, P4 or P5 – use stairs or elevator to JPCH main entrance
     - If you are getting dropped off:
       - **JPCH Entrance at level 5:** (accessible from Hospital or Campus Drive)
         - To drop off patients and visitors, personal vehicles and taxis can enter into the parkade through the parking gate for up to 15 minutes without having to pay for parking. This drop-off option allows patients or visitors easy access to JPCH.
         - Drivers can drop off patients at the Level P5 main doors, but vehicles are not permitted to remain in the driveway
     - If you are taking the bus:
       - **The Parkade Entrance at level 2**: (accessible from Hospital Drive)
         - Can be accessed via foot across from the Law Building – enter P2 doors and go up the stairs to RUH main entrance.
       - **The 'Old Main' entrance:** (features a 1955 sign) this is currently staff only so should be avoided – however, if this is the only accessible entrance to you, please let me know and we may be able to arrange entrance here if RUH Security allows.

- How to enter?
    1. **Make a plan**; Decide which door you are going to come through prior to your visit and please let Kayla know via email or text.
    2. **Arrive early:** arrive a few minutes early for your Asthma Lab appointment to ensure you have enough time to go through any required screening procedures.
    3. **Contact Kayla –** Text or Call Kayla at to notify her when you have arrived at the hospital. She will walk from the lab to the specified entrance to help you get into the hospital. Alternatively, you can call the lab at
    4. **Come prepared:** You may need your Saskatchewan Health Card or some form of ID. We have masks available for you if needed and you are welcome to bring your own as well. You may be required to change your mask into a surgical mask when you enter the hospital. It may be beneficial to bring some hand sanitizer so that you can keep yourself safe while entering. There will be hand sanitizer at entrances and in the Asthma Lab.

2. PPE in the lab:
    - Masks are required at all SHA locations including RUH.
    - We have both surgical and fabric masks available in the lab for your use. Some procedures will require that you do not wear a mask (i.e. spirometry, methacholine challenge testing etc.)
    - Your testing will be performed by a masked lab member.
    - There is hand sanitizer and a sink in the lab – you will be required to wash your hands upon entry and sanitize prior to certain procedures.

3. Sanitization in the lab:
    - We sanitize before and after every lab session to ensure the safety of all lab participants and members.
    - There will be a container that you can set all belongings in to keep your items confined to an easily disinfected space.
    - An exhaust system has been installed in the lab to promote proper air circulation.

4. Waiting periods in the lab:
    - The study will require periods of time that you must wait in the lab between certain tests and procedures.
    - You are welcome to bring items to keep yourself busy – books, study materials, electronics, etc.
    - There will be certain times when you can eat and drink in the lab –as for drinking, water is recommended.

5. Distancing in the lab:
    - The lab is large enough to maintain distancing during most procedures.

- During some procedures, distancing will not be possible and proper PPE of both the lab member and yourself will be required to keep both parties safe.

6. Lab Member Disclosure:
   - Kayla will be the main lab member performing procedures during the study.
   - **Kayla's COVID-19 Risk Disclosure:**

7. Participant Disclosure Form:
   - We ask that you voluntarily fill out the informal COVID-19 Risk Form so that lab members can be informed of your associated risk with being in the lab.
   - This is not an official form by any means. However, it allows lab members to take into consideration any safety risks associated with lab visits during the Pandemic.
   - We ask that you let us know if anything changes regarding your COVID-19 exposure and that you remain open and honest about any COVID-19 risks.
   - The aim of any disclosure is only to keep all other lab members and participants safe during this time.
   - The information you provide will only be available to the lab members: Kayla Cropper, Dr. Beth Davis and Dr. Donald Cockcroft and will not be used for any other purposes.

8. Other participants:
   - All appointments for participants will be spaced out by at least 1-hour to ensure enough time to fully sanitize and prepare the lab.
   - You will be notified if any positive COVID-19 case is identified in the lab and will be given instructions on what to do next.

We appreciate your commitment to the Asthma Lab and willingness to participate during this unprecedented time. If you have any questions about your safety in the lab, please let us know.

Appendix K: BIO #1959 Recruitment Materials

Paws Announcement:

The Asthma Research Lab is conducting a research study to examine the effects of tiotropium bromide on allergen-induced asthmatic responses. Some eligibility criteria include:

- You are 18-65 years old
- You are a non-smoker
- You have controlled or stable asthma
- Your asthma is triggered by allergies (e.g. animals, grass, pollen etc.)

Participation will require 4 visits to the asthma lab. Visits and testing can be done on any day of the week (including weekends).

This is an Anatomy, Physiology and Pharmacology MSc. student project being conducted under the supervision of Dr. Don Cockcroft and Dr. Beth Davis.

You can check out the lab website and obtain a copy of the consent form under the "Studies Needing Participants" tab.

An honorarium will be provided to those individuals who meet all eligibility criteria and participate in the project.

For more information, contact the student researcher:

Kayla Cropper